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PRINCIPAL INVESTIGATOR: Gary P. Zaloga, M.D.

CONTRACTING ORGANIZATION: Clarian Health Partner, Incorporated
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Gary P. Zaloga, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Clarian Health Partners, Incorporated
Indianapolis, Indiana 46206-1367

E-Mail: gzaloga@clarian.org

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The purpose of the breast cancer research-training program is to recruit and train potential future researchers in breast cancer research. We recruited 15 individuals, interviewed eight, and chose five for the program. During the 12-week program, trainees attended seven lectures dealing with research, participated in weekly research meetings, and developed and carried out separate research projects under the supervision of their research mentors. Two projects involved study of the anticancer properties of Ganoderma lucidium (GL), a medicinal mushroom. GL was found to inhibit estrogen receptor signaling in breast cancer cells. Two projects studied the effects of omega-3 long chain polyunsatureated fatty acids (O3FA). O3FA (notably methyl esters of the lipids) were found to inhibit proliferation of an proteolysis induced by breast cancer cells. A fifth project evaluated the effect of C-reative protein (CRP), an important inflammatory molecule upon protein markers of cancer invasiveness. CRP was found to stimulate expression of urokinase plasminogen activator and its receptor. These results provide the basis for ongoing research projects. The results have added significant new information to our understanding of dietary modulation of breast cancer cell proliferation and invasion and provide a basis for futher studies of dietary modulation of breast cancer.

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Introduction

The year 2002 to 2003 was the first year of the Methodist Research Institute's Breast Cancer Research Training Program funded by the US Army Medical Research and Materiel Command. The purpose of the Breast Cancer Research Training Program is to recruit, train, and provide the opportunity to well-qualified college science and premedical students to work on a biomedical research project in breast cancer with a medical researcher in the area of breast cancer. Our goal is to instill in the students a passion for and commitment to breast cancer research so that they might take up breast cancer research as a career. This training is accomplished during the months of May through August.

The report that follows describes the training and research accomplishments of the program, including reportable outcomes such as manuscripts. The body of the report will describe the students and preceptors, and their research projects. The next section will list training accomplishments, and the final narrative section will define the reportable outcomes of the program. The appendix includes reprints of three published studies, abstracts of the projects and printouts of the presentations from Presentation Day, which concludes the program.

Body

Planning for the Breast Cancer Research Training Program begins in November with development of application materials and the start of recruitment procedures. The application due date is generally in mid-February and students are offered positions in late March to early April. Students begin the program the 3rd week of May and the program ends the 2nd week of August with Presentation Day.

Fifteen applicants for the 2003 program expressed an interest in cancer research. We interviewed eight candidates for five positions in the program. Four of the applicants were from underrepresented minorities.

The following list presents the students who were ultimately chosen, the college or university attended, their class status, and their BCRT program preceptor:

Student	Class and Institution	Preceptor
Justin Bell	Senior, Purdue University	Daniel Sliva
Phillip Boykin (minority)	Senior, Rochester Inst of Tech	Frank P. Lloyd, Jr.
Melissa Cain	First Year, Indiana Univ Schl of Med	Carlos A. Labarrere
Laura Sech	Junior, University of Notre Dame	Rafat A. Siddiqui/Gary P. Zaloga
Heidi Yount	Senior, Ohio Northern University	Rafat A. Siddiqui

Training Accomplishments According to the Statement of Work

Task 1: Recruitment of undergraduate students interested in breast cancer research training

- November 18, 2002: Program application updated and information materials prepared for website
- November 18 to November 30, 2002: Information packages with application materials to students, colleges/universities, and community leaders mailed.

Note: We proposed to make follow-up phone calls to college/university biomedical departments and career centers, and to community leaders emphasizing the breast cancer research program; however, at the time this grant was awarded, we had already recruited the candidates for all available positions. Therefore, follow-up calls were not made for the 2003 program. This task will receive greater priority with the 2004 program.

Task 2. Selection of students to participate in the breast cancer research training program

- February 14, 2003: All applications received by February 14, 2003 or with a postmark of February 14, 2003 were considered for the program.
- The Selection and Interviewing Committee chose prospective candidates and interviewed them.

Deviation: We originally proposed to have preceptors interview students. Since that time, the Program Director and the Program Coordinator have decided that rather than the preceptors doing the interviewing, a Selection and Interviewing committee would interview the students. In the past, preceptors had been given three students to interview and would interview the first without interviewing the other two. To create a fairer system in which all students selected for interviewing would have a chance to demonstrate their knowledge and enthusiasm, a committee was convened. For the 2003 program, three of the committee members were faculty on the BCRT project. Interviews were conducted during the 11 days from March 11, 2003 through March 22, 2003.

- March 22, 2003: Interviews with students were completed and committee members chose five students to participate in Breast Cancer Research Training Program and matched them with BCRT preceptors.
- March 24 – March 31, 2003: Selected students were notified of their acceptance to the program.

Task 3: Breast Cancer Research Training Program

- May 19, 2003: Students began the Methodist Research Institute Breast Cancer Research Training Program.
- Students attended seven lectures between May 30, 2003 and July 18, 2003. See appendix for lecture topics and schedule.

- From May 21, 2003 to August 6, 2003, students conducted breast cancer research under guidance of their preceptors. This training included weekly meetings of all student and preceptor participants in the program.
- Students attended a lecture on writing research papers; preceptors guided their students in writing their research papers.
- Preceptors guided students in their preparation of oral presentations; students presented their projects informally to other students at weekly meetings.
- August 7, 2003: All students participated in rehearsal for Presentation Day.
- August 8, 2003: All students delivered oral presentations of their projects to an audience of researchers at Methodist Hospital.
- August 8, 2003: All students turned in their research papers.

Task 4. Evaluation of the Program

- August 12, 2003: Students turned in evaluation forms for the program.
- August 20, 2003: Program Coordinator wrote a final evaluation of the Breast Cancer Research Training Program.
- November 2003: Program Director and Program Coordinator met to review the evaluation.
- Program Director and Program Coordinator developed plans for improvement of the program based on the evaluation.

Deviation: One BCRT preceptor will not be available for the 2004 program because he has left the institution. However, we will continue to train five students using five preceptors. Dr. Thomas Kovala has been added to the faculty.

Key Research Accomplishments

Research Accomplishments

The following paragraphs describe the projects undertaken in the 2003 Breast Cancer Research Training Program and the results of those projects.

Project 1. *Ganoderma lucidum* displays antiestrogenic activity in both MDA-MB-231 and MCF-7 breast cancer cells.

Justin Bell and Dr. Daniel Sliva, Cancer Research Laboratory at the Methodist Research Institute, investigated the anticancer properties of an ancient Asian medicinal mushroom, *Ganoderma lucidum*. Dr. Sliva had previously shown that *Ganoderma lucidum* inhibits proliferation of breast cancer cells. The purpose of this project was to determine whether *Ganoderma* displays estrogenic or antiestrogenic activity in highly invasive breast cancer cells (MDA-MB-231) and in less invasive MCF-7 breast cancer cells. The study found that *Ganoderma lucidum* inhibits estrogen receptor signaling in both MCF-7 and MDMA-MB-231 breast cancer cells.

Project 2. *Breast cancer cells treated with* Ganoderma lucidum *show changes in gene expression.*

Phillip Boykin and Dr. Frank P. Lloyd, Jr., Surgical Oncology, also investigated *Ganoderma lucidum*. The objective of their project was to use microarray analysis to determine whether breast cancer cells treated with *Ganoderma* show differences in gene expression. Results were inconclusive because of RNA degradation after isolation; however, future plans intend to focus more on RNA isolation in preparation for microarray analysis.

Project 3. *The effects of C-reactive protein treatment on uPA and uPAR production by highly invasive breast cancer cells.*

Melissa Cain and Dr. Carlos A. Labarrere, Experimental Pathology Laboratory at Methodist Research Institute, examined whether C-reactive protein (CRP), an acute-phase protein with immunological functions, promotes cell growth in highly invasive MDA-MB-231 breast cancer cells through upregulation of urokinase plasminogen activitor (uPA) and its receptor (uPAR). Their study found that CRP promotes increased secretion of uPA by highly invasive breast cancer cells and promotes upregulation of uPAR on the cell membrane of highly invasive breast cancer cells.

Project 4. *Analysis of commercial fish oil supplements and their efficiency in inhibiting metastasis and promoting apoptosis of breast cancer cells.*

Laura Sech and Ds. Rafat A. Siddiqui and Gary P. Zaloga, Cellular Biochemistry Laboratory at Methodist Research Institute, studied the anticancer effects of omega-3 fatty acids in fish oil. Ms. Sech's project examined seven commercial preparations of omega-3 lipids for differences in chemical composition, the quantity of omega-3 fatty acids present, and their anticancer activity in MDA-MB-231 breast cancer cells. Her analysis found that significant compositional differences exist between the different preparations, and that the different preparations exhibited widely variable anticancer effects. This study also found that fatty acid methyl esters had the most potent anticancer effects.

Project 5. *Role of omega-3 fatty acids in the prevention of cancer-induced muscle proteolysis.*

Heidi Yount and Dr. Rafat A. Siddiqui, Cellular Biochemistry Laboratory at Methodist Research Institute, also studied omega-3 fatty acids (O3FAs); however, they examined the role of O3FAs in preventing muscle proteoloysis induced by breast cancer. The hypothesis of the study was that O3FAs, primarily docosahexaenoic acid, provide protection against tumor-induced muscle wasting by inhibiting activation of calpains. The results of the study suggest that MDA-MB-231 breast cancer cells release soluble proteolysis-inducing factors that induce proteolysis by activating caplain enzymes. Omega-3 fatty acids inhibit the calpain-associated proteoloysis pathway. Thus, omega-3 lipids may help prevent body-wasting associated with cancer.

Reportable Outcomes

Manuscripts:
The following manuscripts were produced as a result of the research completed by students during the 2003 Summer Student Research Program. Students' names are in bold.

Preceptors: Rafat Siddiqui and Gary Zaloga:

1. Siddiqui, R., **Sech, L.,** Zerouga, M., Castillo, A., Zaloga, G., Stillwell, W. Analysis of commercial omega-3 fatty acids supplements and their efficiency in inhibiting cell adhesion and promoting apoptosis in breast cancer cells. ***J Altern Compl Med.*** *(submitted).*

2. Siddiqui, R., Shaikh, S., **Sech, L., Yount, H.,** Stillwell, W., Zaloga, G., Omega 3-fatty acids: health benefits and cellular mechanisms of action. ***Rev Curr Med Chem.*** *(in press).* (See galley proof in appendix.)

3. **Yount, H.**, Siddiqui, R. (2004) Role of omega 3-fatty acids in the prevention of cancer induced muscle proteolysis. ***Am Chem Soc.*** *(in press).*

Abstracts:

Preceptor: Rafat Siddiqui:
Yount, H., Siddiqui, R. (2004) Role of omega 3-fatty acids in the prevention of cancer induced muscle proteolysis. ***Am Chem Soc.*** 2004, Abstract #CHD242.

Student abstracts published in the 2003 Summer Student Research Program Presentation Day Program (see appendix).

Presentations:
Student presentations presented at the 2003 Summer Student Research Program Presentation Day (see appendix).

Conclusions
We successfully recruited and selected five individuals to participate in the 12-week Breast Cancer Research Training Program at the Methodist Research Institute. Trainees attended a series of lectures dealing with research design, statistics, ethics, and research reporting. Trainees developed and worked on individual research projects under direct supervision of a mentoring researcher. Each trainee significantly contributed to larger research projects dealing with breast cancer. Four of the five projects dealt with dietary modulation of breast cancer proliferation and invasion, and involved evaluation of the effects of Ganoderma lucidium (a mushroom) and omega-3 long chain fatty acids. Both of these compounds have been found to have anticancer activities in previous studies at the institute. The fifth project evaluated the effects of C-reactive protein, a proinflammatory compound, upon breast cancer cells. The knowledge obtained in these studies contributes to our knowledge of dietary modulation of breast cancer cell growth and invasion and results are in the process of being submitted for publication. These results will help determine future areas of research.

APPENDICES

Student Abstracts from the 2003 Presentation Day Program

Student Presentations Presented at the 2003 Presentation Day

Galley Proof: Siddiqui, R., Shaikh, S., Sech, L., Yount, H., Stillwell, W., Zaloga, G., Omega 3- fatty acids: health benefits and cellular mechanisms of action. *Rev Curr Med Chem. (in press).*

Justin Bell
Purdue University

Preceptor: Daniel Sliva, PhD
Cancer Research Laboratory,
Methodist Research Institute

GANODERMA LUCIDUM DISPLAYS ANTIESTROGENIC ACTIVITY IN BOTH MDA-MB-231 AND MCF-7 BREAST CANCER CELLS

Introduction In 2001, the American Cancer Society provided an annual estimate of 192,200 new breast cancer cases in females, as well as 1,500 new cases in males. The complex and mysterious nature of breast cancer has brought about multiple treatments ranging from conventional pharmaceutical drugs to herbal therapies. One of these ancient herbs of interest is the basidiomycetous fungi *Ganoderma lucidum,* which has been used for centuries in traditional Asian medicine to cure everything form hypertension to cancer. Recently *Ganoderma lucidum* has demonstrated the potential to suppress the motility of highly invasive breast and prostate cancer cells. This mechanism of inhibition seems to parallel that of the antiestrogenic drugs or phytoestrogens, which act as selective estrogen receptor modulators (SERM) and can inhibit proliferation of breast cancer cells.

Objective The aim of this study was to determine whether *Ganoderma lucidum* displays either estrogenic or antiestrogenic activity in MCF-7 and MDA-MB-231 breast cancer cells.

Methods To determine the estrogenic or antiestrogenic activity of *Ganoderma lucidum,* we assessed the activity of the estrogen receptor (ER) by utilizing an estrogen response element ERE-CAT assay. Both breast cancer cells MDA-MB-231 and MCF-7 were transiently transfected with 5 μg ERE-CAT reporter construct and 3 μg β-galactosidase expression vector pCH110. Two different experiments were then carried out. One involved both cell types being treated with solely *Ganoderma lucidum* at varying concentrations. The second experiment involved treatment with both *Ganoderma lucidum* and β-estradiol, which is a potent estrogen. In both experiments, cell extracts were then prepared followed by both the β-gal assay and the CAT assay.

Results Both experiments revealed that *Ganoderma lucidum* inhibits estrogen receptor signaling in MCF-7 (ERα-positive, ERβ-positive) and MDA-MB-231 (ERα-negative, ERβ-positive) breast cancer cells.

Conclusion *Ganoderma lucidum* displays antiestrogenic activity in both noninvasive and invasive breast cancer cells. This evidence sheds some light on the future role that *Ganoderma lucidum* may play in the prevention or treatment of breast cancer through the inhibition of cancer cell proliferation.

Justin Bell's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the US Army Medical Research and Materiel Command.

Justin will be a super-senior (5th yr) at Purdue University-Lafayette this coming fall. He is majoring in both biology and secondary education, along with a minor in chemistry. Justin wants to thank both his preceptor and lab partners for such an excellent summer experience.

Phillip Boykin
Rochester Institute of Technology

Preceptor: Frank P. Lloyd, Jr., MD
Surgical Oncology

BREAST CANCER CELLS TREATED WITH *GANODERMA LUCIDUM* SHOW CHANGES IN GENE EXPRESSION

Introduction Microarray was the method used in this research. This technique allows one to compare the differential gene expression of contrasting cell types. In this experiment, the gene expression of two types of breast cancer cells treated with *Ganoderma lucidum* was determined. The gene expression of the *Ganoderma lucidum* treated cells was then compared to the gene expression of breast cancer cells not treated with *Ganoderma lucidum*. This comparison of *Ganoderma lucidum*-treated and untreated breast cells sought to identify certain expressions that showed significant up-regulation or down-regulation. For this experiment, two types of breast cancer cells were used. The first cell type was MCF-7, which is a non-invasive form of breast cancer. The second cell type is MDA-MB-231 and is an invasive form of breast cancer. Both MCF-7 and MDA-MB-231 cells were treated with *Ganoderma lucidum*. The mushroom, *Ganoderma lucidum*, has been used in East Asia for centuries to combat many diseases such as hepatitis, bronchitis, and cancer. *Ganoderma lucidum* has been shown to stop metastasis and adhesion of cancer cells (Daniel Sliva, Ganoderma lucidum suppresses motility of highly invasive breast and prostate cancer cells, Sept. 2002). The objective of this experiment is to use the method of microarray to see if breast cancer cells treated with *Ganoderma lucidum* express differences in gene expression.

Methods Both types of breast cancer cells, MCF-7 and MDA-MB-231, were treated with *Ganoderma lucidum* for 24 hours and then allowed to grow free of the mushroom treatment for another 24-hour period. Next, RNA was isolated from the treated cells. RNA was also isolated from control samples of MCF-7 and MDA-MB-231 not treated with *Ganoderma lucidum*. The *Ganoderma lucidum*-treated cells were labeled with a red dye, Cy5. The control cells were labeled with a green dye, Cy3. Both treated and control cells were then hybridized onto a VARI human microarray slide in the following way: the MDA-MB-231–treated and control cells were hybridized onto one slide and the MCF-7–treated and control cells were hybridized on another slide. Next, the two slides were scanned by a microarray laser, which displayed a visual of the gene expression.

Results There is differential gene expression in breast cancer cells treated with *Ganoderma lucidum,* as evident from published data. Unfortunately with our experiment, all the isolated RNA samples taken from both treated and control cell types were degraded; therefore, the microarray didn't yield any substantial data.

Conclusion RNA isolation is a delicate step in producing a microarray and once RNA is isolated from a cell it can't stay intact for very long. Replication of this experiment with special emphasis placed on RNA isolation is the plan as of now.

Phillip Boykin's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the US Army Medical Research and Materiel Command.

Phillip Irvin Boykin is currently a 4th year biology major at Rochester Institute of Technology with minors in anthropology and sociology. He is a premedical student and intends to obtain a combined biology/MBA degree through a five-year program at his undergraduate institution. Compassion is his most treasured trait while family, science, and music are what give his life meaning.

Melissa Cain
Indiana University Laboratory, School of Medicine

Preceptor: Carlos Labarrere, MD
Experimental Pathology

Methodist Research Institute

THE EFFECTS OF C-REACTIVE PROTEIN TREATMENT ON UPA AND UPAR PRODUCTION BY HIGHLY INVASIVE BREAST CANCER CELLS

Introduction C-reactive protein (CRP) is an acute-phase protein that has many known immunological functions. Elevated plasma CRP levels have been associated with numerous disease states, including malignancy. This correlation between C-reactive protein and cancer is not well understood. Although this is not well understood, it is known that urokinase plasminogen activator (uPA) and its receptor (uPAR) are involved in cancer cell adhesion, migration, and invasion. We hypothesized that C-reactive protein promotes highly invasive breast cancer cell growth through uPA and uPAR upregulation by nuclear factor kappa B (NF-κB) activation.

Methods MDA-MB-231 (a highly invasive breast cancer cell line) cells were grown using media with serum until they were 80-90% confluent. At this time, the media was removed and replaced with serum-free media. In this starved state, the experimental cells were treated with serum-free media supplemented with 0 μg/ml, 10 μg/ml, and 50 μg/ml of C-reactive protein and incubated for 24 hours, 4 hours, and 1 hours. The media was removed after incubation and concentrated by centrifugation filtration. Western blots were performed on the concentrated media using anti-uPA and anti-uPAR antibodies. The MDA-MB-231 cells were used to prepare cell extracts and cytospins. The cell extracts were run on gels, and Western blots were completed using anti-uPA and anti-uPAR antibodies. These cell extracts were also used to perform NF-κB enzyme linked immunosorbent assays. The cytospins for each incubation time and concentration were immunohistochemically labeled for uPA and uPAR using fluorescently conjugated antibodies.

Results For the cells that had been incubated in 10 μg/ml and 50 μg/ml of C-reactive protein, a qualitative increase in uPA was observed on the Western blots of the media, as compared to the cells that had not been treated with CRP. The cells that had been treated with 50 μg/ml CRP showed a greater increase in uPA than did the cells that had been treated with 10 μg/ml. Using the Western blot technique, no uPAR was detected in the media from either the control cells or the cells that had been treated with C-reactive protein.

A qualitative increase in uPA was also observed in the cells treated with 10 μg/ml and 50 μg/ml CRP in the cytospin, as compared to the cells that had not been treated. The longer the cells were incubated in the CRP, the greater the increase in uPA. There was also a qualitative increase in uPAR observed in the cells treated with both 10 μg/ml and 50

μg/ml of C-reactive protein. A correlation between length of incubation and increase in uPAR was also detected.

Conclusions C-reactive protein promotes increased secretion of urokinase plasminogen activator by highly invasive breast cancer cells. CRP also promotes the upregulation of the urokinase plasminogen activator receptor on the cell membrane of highly invasive cell membranes. The mechanism of upregulation warrants further investigation.

Melissa Cain's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the US Army Medical Research and Materiel Command.

In the fall, **Melissa** will be starting her second year of medical school at Indiana University School of Medicine's Northwest Center for Medical Education. She graduated in May 2002 from Indiana State University with a B.S. in mathematics and a life science minor. As a physician, Melissa would like to specialize in anesthesiology, surgery, neurology, emergency medicine, or perhaps a combination thereof.

Laura Sech
University of Notre Dame

Preceptor: Rafat Siddiqui, PhD
Gary P. Zaloga, MD
Cellular Biochemistry Laboratory,
Methodist Research Institute

ANALYSIS OF COMMERCIAL FISH OIL SUPPLEMENTS AND THEIR EFFICIENCY IN INHIBITING METASTASIS AND PROMOTING APOPTOSIS OF BREAST CANCER CELLS

Countless studies have shown the beneficial impact that the essential omega-3 fatty acids have in regard to heart disease and cancer, two of the leading causes of death in America each year. Despite the fact that repeated analysis of omega-3 fatty acids has consistently shown that they suppress heart failure and reduce tumor growth in laboratory experiments, the mechanism by which they affect these illnesses remains unclear. Thus, the importance of omega-3 supplementation, or eating foods rich in omega-3 such as fish or flax seeds, has been understated largely due, in part, to the Food and Drug Administration's refusal to recognize omega-3 products as potential treatments for heart failure and/or cancer. However, as a result of Internet access, many people have begun to become informed about the benefits of omega-3 fatty acids, and a large market has become readily accessible for purchasing omega-3 supplements. Therefore, it is important that omega-3 supplements be analyzed and tested for their efficiency in treatment as well as noticing differences between the brands.

In this study specifically, seven different brands of omega-3 supplements were chosen (Sundown Flax Seed Oil, Sundown Cod Liver Oil, Sundown Fish Oil, Puritan's Pride Super EPA, Nature Made Fish Oil, Member's Mark Fish Oil, and Sigma-Aldrich Fish Oil), and tests were performed to determine any chemical composition differences as well as the quantity of omega-3 present using techniques such as thin layer chromatography (TLC) and gas chromatography (GC).

TLC results show that the majority of fatty acids are present as triglycerides (60%-90%). GC analysis measured the amount of omega-3 fatty acids present in each sample as well as the presence of other polyunsaturated and saturated fats that are not represented on nutrition labels. According to these results, flaxseed oil contains the largest percentage of omega-3 per capsule (55%); however, this large quantity is in the form of alpha-linolenic acid and is not known to have the same biologically beneficial effects as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), which are both present in the other samples. Furthermore, GC analysis shows slight differences between the quantities of omega-3 present in the capsule versus the amount that is marketed by the manufacturer with the exception of the Sigma-Aldrich fish oil.

Based upon GC data, the presence of all significant amounts of polyunsaturated fatty acids (PUFAs) can be used to calculate the unsaturation index for each sample; this is an important characteristic, especially when considering the peroxidation levels of each sample. Thus, after discovering the oxidation potential for each sample, a lipid hydroperoxide assay was performed using a protocol outlined by the Cayman Chemical

Company, and the results were correlated to display the quantity of lipid peroxidation per gram of omega-3 fatty acid present. Both the Sundown flaxseed oil and Puritan Pride Super EPA brands showed the lowest amount of lipid peroxidation per grams of omega-3 while the cod liver oil showed the highest amount. The two brands, Nature Made and Member's Mark, even though supplemented with Vitamin E, had average levels of lipid peroxidation in comparison to the other brands. Using these results, we hope to find a correlation between the unsaturation index and lipid peroxidation concentration of the samples.

Finally, after initial analysis of chemical composition, unsaturation index, and lipid peroxidation, the fish oil samples were incubated for 2, 4, and 24 hours with MDA breast cancer cells using a vitronectin cell adhesion assay. After 4 hours, the Puritan Pride Super EPA brand showed the least amount of cell adherence (less than 10%), and after 24 hours of incubation, less than 1% adherence. Following the 24-hour incubation period, cells were treated with WST-1 to calculate for cell viability. Results show that the Puritan Pride brand showed the lowest percentage of cell viability whereas the Nature Made brand showed the highest percentage. These results indicate that after prolonged exposure to fish oil, the Puritan Pride brand appears most toxic for the MDA cells.

Although it was our hope that the analysis of the seven brands would show no significant differences in regard to their content of peroxidation and ability to prevent cell adhesion, it appears that omega-3 supplements are not uniform and must, therefore, be processed differently. Moreover, it would be premature to claim that a particular brand is "most effective," especially when we consider the countless number of brands that are currently available. However, this experiment displays a favorable outcome for the Puritan Pride Super EPA supplement.

Laura Sech's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the US Army Medical Research and Materiel Command.

Laura is about to embark upon her last year as an undergraduate student at the University of Notre Dame. GO IRISH!!!!! For those of you who doubt the superiority of the Notre Dame football team, she will at this moment make the claim that the 2003 season will go down in history as a year of national championship victory. If anyone wants to challenge this claim, Laura will be happy to speak with you outside. On a more serious note, Laura wants to thank Dr. Siddiqui for being the best preceptor in the entire world and teaching her the finer phrases of Urdu; she is sure that they will come in handy later in life. After graduating from Notre Dame (Laura believes in thinking positively), she will hopefully attend medical school in someplace outside of the Midwest where she has lived for 21 years (preferably Puerto Rico). If the admissions officers decide that Laura is not suited to pursue a career in medicine, she will more than likely move to Mexico and become a professional salsa dancer. To close, Laura would also like to bid a most heartfelt farewell to the friends that she has made this summer. She exclaims, "All of you are smart, beautiful, and wonderful people!!" She also wants to send her love out to her parents who are not in attendance today but probably would feel left out if they were not included in this lengthy paragraph.

Heidi Yount
Ohio Northern University

Preceptor: Rafat Siddiqui, PhD
Cell Biochemistry Laboratory,
Methodist Research Institute

ROLE OF OMEGA-3 FATTY ACIDS IN THE PREVENTION OF CANCER-INDUCED MUSCLE PROTEOLYSIS

Growth of cancer causes many detrimental effects on the host system. One of the contributing factors in cancer mortality is loss of lean body mass due to muscle wasting (cachexia). Cachexia occurs in more than two thirds of patients who die with advanced cancer and is estimated to be the cause of death for approximately 22% of cancer patients.[1] Muscle hypercatabolism is caused via three proteolytic pathways: the calpain (calcium-dependent neutral protease) pathway, the ATP-ubiquitin proteasome pathway, and the lysosomal pathway. We hypothesized that tumor growth releases soluble proteolysis-inducing factor(s) that induce proteolysis by activating calpain enzymes and further hypothesized that omega-3 fatty acids, primarily docosahexanoic acid (DHA), provide protection against tumor-induced muscle wasting by inhibiting activation of calpains.

To test our hypothesis, we investigated calpain-mediated proteolytic pathway involvement using an in vitro system, including skeletal muscle and cardiac muscle cells. Skeletal muscle was derived from differentiation of L8 myoblast cells, whereas cardiomyocytes were isolated from neonatal rats. Muscle proteolysis in these cells was induced by soluble extracts of different cancer cells, including leukemia (Jurkat), breast cancer (MDA-231-MB), cervical cancer (HeLa), and colon cancer (T84) cells, along with their growth media. The proteolysis was analyzed by measuring the release of ^{3}H-tyrosine of prelabeled cells. The results indicate that the growth media from cervical cancer cells induced proteolysis by 35% in skeletal muscle cells. Similarly, the soluble cell extracts of these cells also induced proteolysis by 24%. Growth media from breast cancer and leukemic cells induced proteolysis by 95% and 25% respectively. The soluble cell extracts of these cells have no effect on the skeletal muscle cells. The results involving the colon cancer cells have not been finalized yet. In cardiac cells, breast cancer, cervical cancer, and leukemic cells also induced proteolysis. In comparison to control conditions, growth media from breast cancer cells induced a 37% increase in proteolysis. Growth media from leukemic cells induced a 26% increase in proteolysis, and the soluble extracts from these cells induced a 31% increase in proteolysis. Growth media from cervical cancer cells induced proteolysis by 43%, and the soluble extracts of these cells induced proteolysis by 30%.

Following the calpain study, we then investigated whether dietary supplementation of ω-3 fatty acid has any effect on breast cancer cell (MDA-231-MB) growth and tumor-induced muscle proteolysis. Breast cancer cells were implanted in nude mice, which were fed a corn oil-rich diet (Group 1, 1:18 ω-3 FA:ω -6 FA), a fish oil-rich diet (Group 2, 2.6:1 ω-3 FA:ω-6 FA), or a balanced mixture of corn oil and fish oil (Group 3, 1:1 ω-3

FA:ω-6 FA) for 3 weeks prior to tumor implantation. Following implantation, the diets were continued for an additional 3 weeks. The data indicates that growth tumors were reduced by 50% and 30% in Groups 2 and 3, respectively, compared to those of Group 1. The cardiac and skeletal muscle tissues from these animals were then analyzed for calpain activities. In the cardiac muscle tissues, it was found that the activities of calpain were inhibited by 171% and 72% in ω-3 FA–rich and balanced diet-fed animals, respectively, compared to the that of ω-6–rich diet-fed animals. The results were similar for the skeletal muscle tissue. Calpain activities were inhibited by 35% and 134% in ω-3 FA–rich and balanced diet-fed animals, respectively, than that of ω-6–rich diet-fed animals.

We are further testing our in vivo results in an isolated cellular system. At present, we are in the process of treating skeletal muscle and cardiac muscle cell to DHA prior to induction of muscle proteolysis by soluble tumor factors. Results are not completed for this experiment, but we expect to see a reduction in proteolysis in the cells treated with DHA. Overall, this study suggests that cancer cells release soluble proteolysis-inducing factor(s) that induce proteolysis by activating calpain enzymes. Omega-3 FA inhibits the calpain-associated proteolysis pathway and its treatment therefore offers a preventive effect against body-wasting associated with cancer.

1. Alvarez, Belen, et al. *Tumor necrosis factor-α exerts interleukin-6-dependent and independent effects on cultured skeletal muscle cells*. Biochimica and Biophysica Acta. 1542 (2002): 66-72.

Heidi Yount's participation in the Breast Cancer Research Training Program was supported by grant #BC020180 from the US Army Medical Research and Materiel Command.

Heidi is a senior biochemistry major at Ohio Northern University. Upon graduation, she plans to attend medical school.

Determination of estrogenic/anti-estrogenic activity of *Ganoderma lucidum* in breast cancer cells.

Cancer Research Lab

Justin Bell

Preceptor: Dan Sliva, Ph.D.

Justin Bell's participation in the Breast Cancer Research Training Program was supported by grant # BC020180 from the Department of the Army Medical Research and Materiel Command.

What's the PROBLEM??

- 1 out of 8 women will develop BCa.
- Estimated 192,200 new cases in females and 1,500 in men in US alone, with 40,600 expected deaths due to breast cancer.

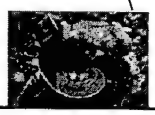
(National Cancer Institute)

The Overall Idea

STOP CANCER CELL PROLIFERATION, MIGRATION, ADHESION, AND INVASIVENESS!!

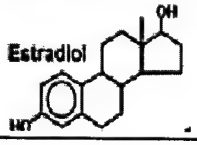
Background: *Ganoderma lucidum*

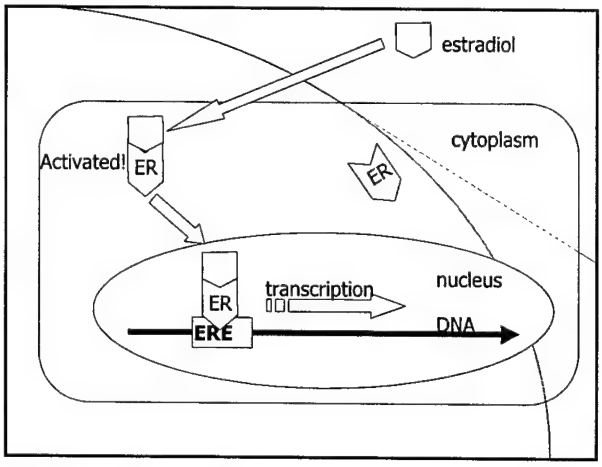
- Known anti-tumor effects due to triterpenes and polysaccharides.
- Potential to suppress motility of highly invasive breast and prostate cancer cells. (Sliva 2002)



Background: Estrogen

- Hormone implicated in the development and progression of breast cancer. (Khan 1998)
- Estradiol is a potent estrogen type hormone produced in the body.

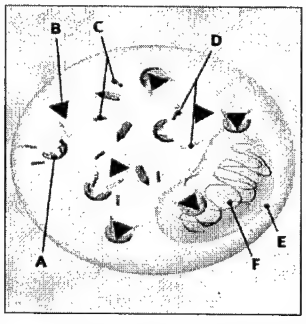




HYPOTHESIS

Ganoderma lucidum has phytoestrogenic activity and is a selective estrogen receptor modulator (SERM).

Hypothesized Role of G.L.

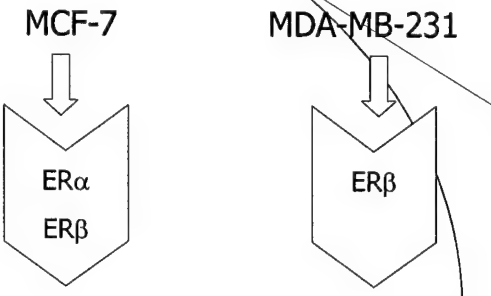


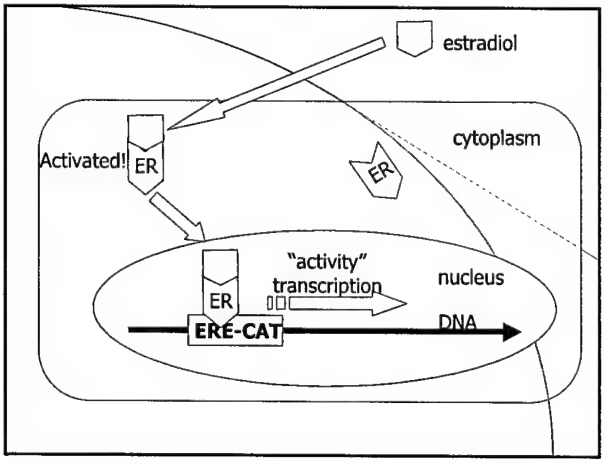
A estrogen receptor
B ***G.L.*** **?**
C estrogen helper proteins
D ***G.L.*** helper proteins **?**

Experimental Method

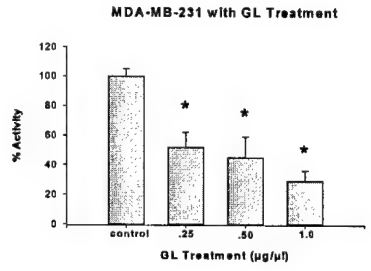
1. Transfect MCF-7& MDA-MB-231with ERE-CAT and β-Gal.
2. Treat cells with G.L. and then G.L. + Estradiol.
3. Assess ERE activity by measuring CAT activity of treated cells.

Breast Cancer Cell Types





Results: GL Treatment



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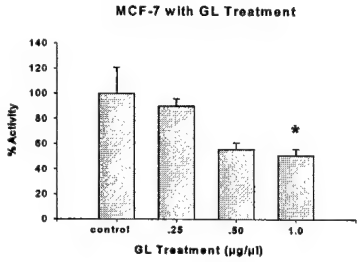
Results: GL Treatment



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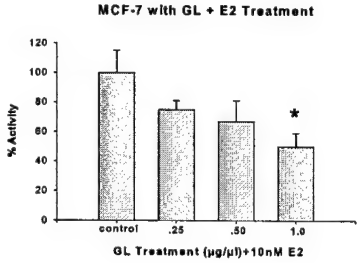
Results: GL Treatment

	ER % Activity	
	MDA-MB-231	MCF-7
Control	100 ± 7	100 ± 19
1.0 µg/ml	44 ± 6	49 ± 6

No significant difference at 1.0 µg/ µl.

Results: GL + E2 Treatment



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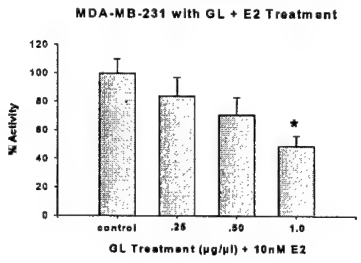
Results: GL + E2 Treatment



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Results: GL + E2 Treatment

	ER % Activity	
	MDA-MB-231	MCF-7
Control	100 ± 13	100 ± 11
1.0 μg/ml	70 ± 15	60 ± 10

No significant difference at 1.0 μg/ μl.

Conclusions

Ganoderma lucidum displays anti-estrogenic activity in both MCF-7 and MDA-MB-231 breast cancer cell types.

Implications/Future Research

- Possible role of *Ganoderma lucidum* as adjuvant or preventative therapy.
- Treat stable transfected cell line containing various combinations of the estrogen receptor with *Ganoderma lucidum.*

Appreciation Slide!!

Breast Cancer Cells treated with *Ganoderma lucidum* show Changes in Gene Expression

Presented by:
Phil Boykin

Preceptor
Frank Lloyd Jr., MD

Phil Boykin's participation in the Breast Cancer Research Training Program was supported by grant # BC020180 from the Department of the Army Medical Research and Materiel Command.

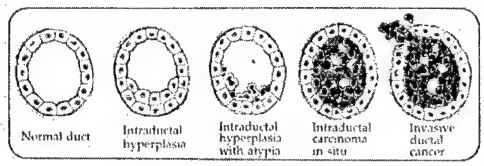
Outline of Presentation

- Introduction
- Hypothesis
- Experimental Outline
- Method
- Discussion of Results

Introduction

- Breast Cancer two cell types
  - A) MDA-MB-231
  - B) MCF-7

Introduction



Introduction

Ganoderma lucidum:

Has been used since ancient times in East Asia in therapies for different diseases (hepatitis, bronchitis, and cancers) (Sliva 603)

Introduction

Ganoderma lucidum Functions (Sliva 603)

Believed to stop cancer cell

- metastasis
- adhesion cells

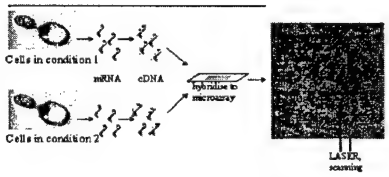
Hypothesis

- Treatment of highly invasive (MDA-MB-231) and less invasive (MCF-7) breast cancer cells with G. *lucidum* will cause a change in gene expression.

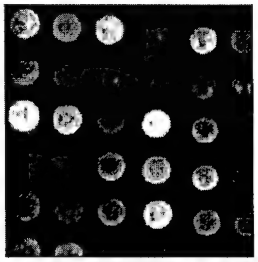
Experimental Outline

Control (G.L. Untreated)	Experimental (G.L. Treated)
MCF-7	MCF-7
MDA-231	MDA-231

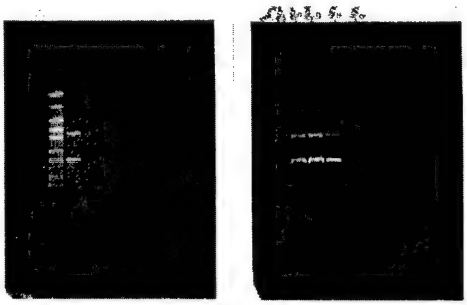
Microarray Simplified

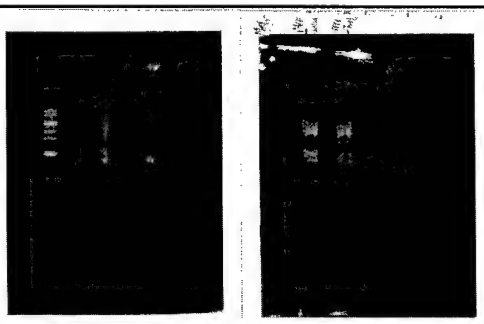


Method: Microarray



MDA & MCF RNA at MRI

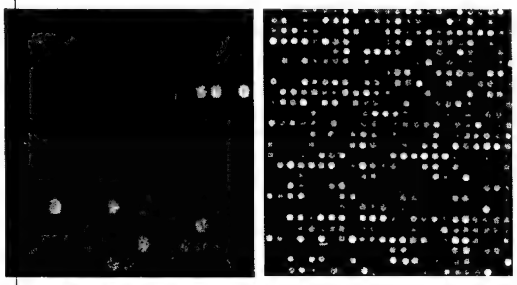


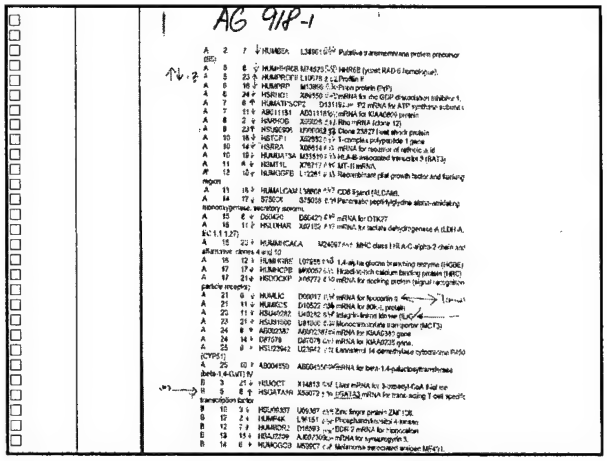


MDA & MCF at VARI

How it turned out

How it should look





Works Cited

Sliva, Daniel. *Ganoderma lucidum* suppresses motility of highly invasive breast and prostate cancer cells. Indianapolis: Elsevier Science, 2002. 603.

Acknowledgements

- Veronika Slivova
- Daniel Sliva, PhD.
- Tatiana Valachovicova
- David Jiang
- Justin Bell
- Michael Sjoding

Questions?????????

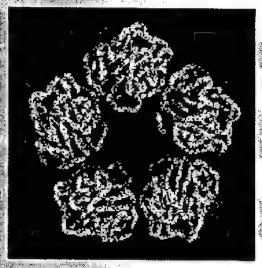
Effects of C-reactive protein treatment on uPA and uPAR expression by highly invasive breast cancer cells

Melissa Cain
Carlos Labarrere, M.D.
Methodist Research Institute

Melissa Cain's participation in the Breast Cancer Research Training Program was supported by grant # BC020180 from the Department of the Army Medical Research and Material Command.

C-reactive Protein

- 115 kDa molecule
- Produced in the liver
- Acute Phase Protein
- Functions
  - Activates complement pathway
  - Binds and modulates behavior of phagocytic cells
  - Other functions not well understood
- "Normal" serum level is <1 µg/ml



*Grunhough, T.J. and coworkers, Keele University U.K., Image copyright Keele University http://us.expasy.org/spotlight/articles/spt1030/html

Serum CRP levels associated with disease

< 1 µg/ml	1-10 µg/ml	>10 µg/ml
Common Cold	Mucosal Infection	Acute Bacterial Infection
Vigorous Exercise	Myocardial Infarction	Major Trauma
Pregnancy	Rheumatic Disease	Systemic Vasculitis
Angina	Pancreatitis	Transplant Vasculopathy
Seizures	Malignancies	

How is CRP related to malignancies?

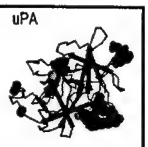
- In vivo, CRP treatment after the introduction and removal of mouse mammary cell adenocarcinoma inhibits growth and metastasis[1]
- In vitro?
  - No studies have been published

[1] Krest, et al. "Inhibition of Mouse mammary Adenocarcinoma (EMT6) Growth and Metastases in Mice by a Modified Form of C-Reactive Protein

Hypothesis

- C-reactive protein promotes highly invasive breast cancer cell growth through uPA and uPAR up-regulation. Nuclear factor kappa B (NF-κB) activation could mediate this up-regulation.

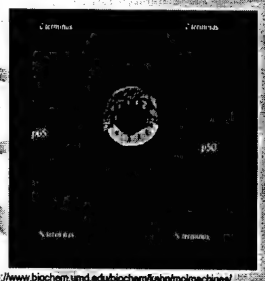
Urokinase Plasminogen Activator (uPA) and urokinase Plasminogen Activator Receptor (uPAR)



http://www.biochem.ucl.ac.uk/bsm/pdbsum/1c5w/racel.html

- uPA is
  - a serine protease
- uPA/uPAR is involved in cell
  - Adhesion
  - Migration
  - Invasion
- Gene expression is NF-κB dependent
- Increasing NF-κB activation up-regulates uPA and uPAR

Nuclear Factor-Kappa B (NF-κB)

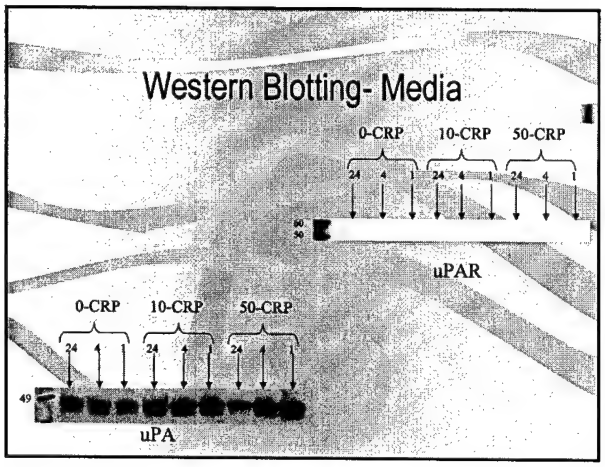


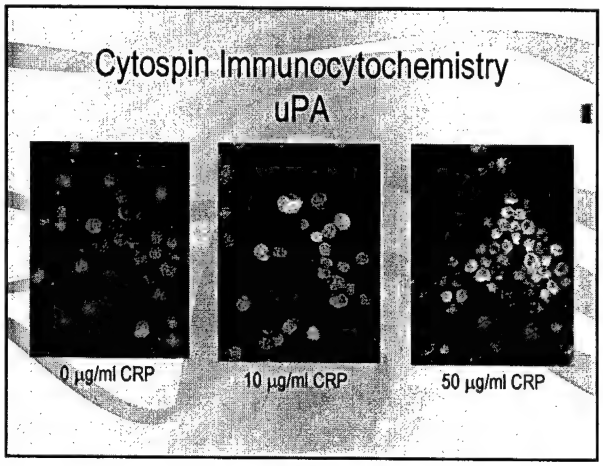
http://www.biochem.umd.edu/biochem/kahn/molmachines/enhancesomes/NFkB.html

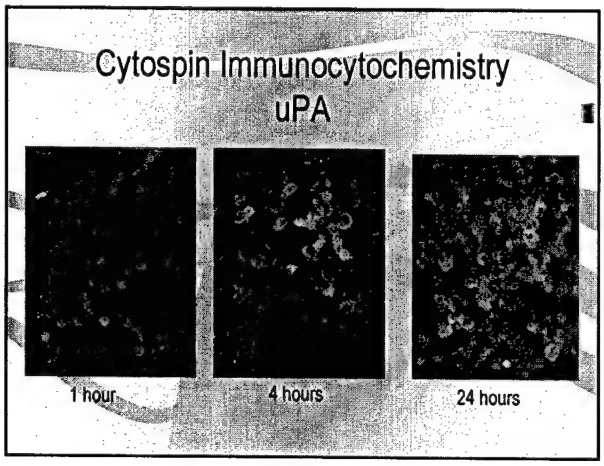
- Transcription Factor
- Normally found in cytoplasm in inactive form
  - Bound to IκB-α
- Active form of NF-κB triggers transcription of several different genes

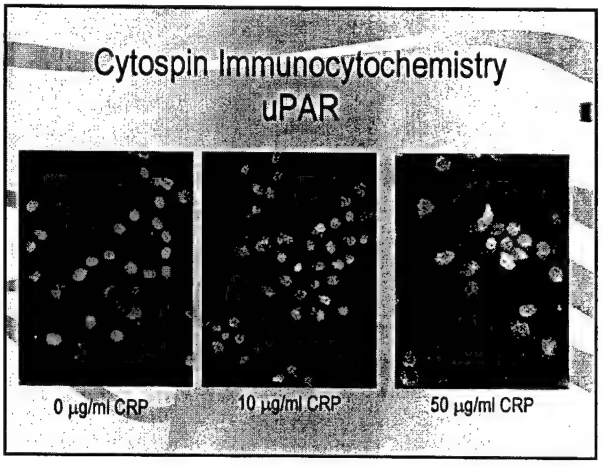
Methods

- Cell Culture
  - treat with 0 μg/ml, 10 μg/ml, and 50 μg/ml of CRP
  - incubate for 1, 4, and 24 hrs
- Western Blotting
  - determine the presence of uPA and uPAR in culture media
- Enzyme Linked Immunosorbent Assay (ELISA)
  - evaluate the activity of NF-κB
- Cytospin and Immunocytochemistry
  - detect the presence of uPA and uPAR

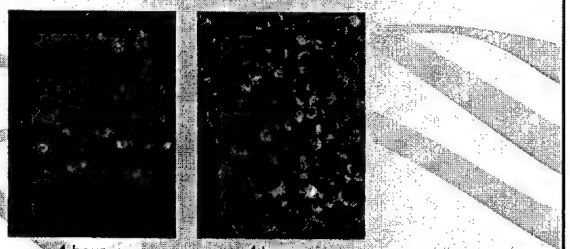

Western Blotting- Media
0-CRP
10-CRP
50-CRP
24
4
1
uPAR
uPA

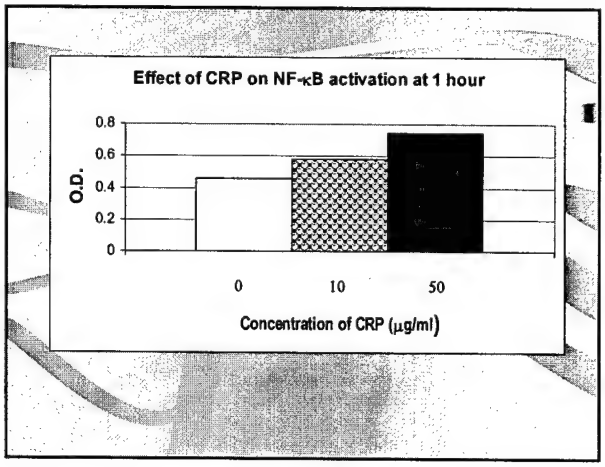

Cytospin Immunocytochemistry
uPA
0 µg/ml CRP
10 µg/ml CRP
50 µg/ml CRP


Cytospin Immunocytochemistry
uPA
1 hour
4 hours
24 hours


Cytospin Immunocytochemistry
uPAR
0 µg/ml CRP
10 µg/ml CRP
50 µg/ml CRP

Cytospin Immunocytochemistry uPAR



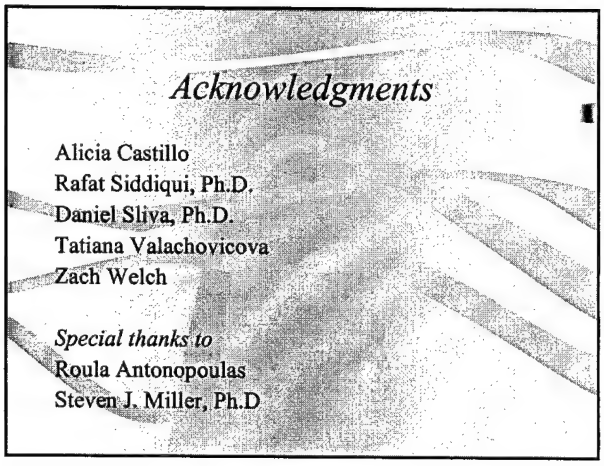


Conclusion

- In highly invasive breast cancer cells, CRP promotes
  - secretion of uPA
  - up-regulation of uPAR
- CRP increases NF-κB activation

Future Studies

- Cell Extract
  - Western Blotting
    - uPA
    - uPAR
    - Phosphorylated IκB-α
- Cytospin
  - uPA/uPAR complex
- Fluorescent Activated Cell Sorter (FACS)
- Repeat NF-kB ELISA
- Gel Electrophoretic Mobility Shift Assay (GEMSA)
- Other transcription factors (AP-1)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Zach Welch
Special thanks to
Roula Antonopoulas
Steven J. Miller, Ph.D

Analysis of Commercial Fish Oil Supplements and their Efficiency in Inhibiting Metastasis and Promoting Apoptosis of Breast Cancer Cells

Laura Sech

Rafat Siddiqui, PhD; Gary Zaloga, MD

Laura Sech's participation in the Breast Cancer Research Training Program was supported by grant # BC020180 from the Department of the Army Medical Research and Materiel Command.

Why Study Fish Oil?

- Rich in essential omega-3 fatty acids
- Has yielded successful results in lab experiments for suppressing heart failure and reducing tumor growth
- Has created a large market for supplementation that is readily accessible in grocery stores and on the internet

Omega-3 in the News

*** Omega 3 and ADHD**

- Scientific evidence has shown that ADHD individuals have a significantly lower percentage of Docosahexaenoic acid in the blood.

*** Omega-3 and the FDA**

- Petition for labeling food and/or food supplements that contain Omega-3

Omega-3 and Cancer Treatment
-Prosure
-combats tumor induced weight loss

OUR CANDIDATES

1- Sundown Flax Seed Oil (FS) 

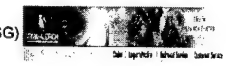
2-Sundown-Cod Liver Oil (CL) 

3-Sundown Fish Oil (SD) 

4-Puritan's Pride-Super EPA (PP)

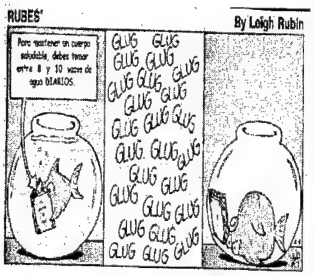
5-Nature Made Fish Oil (NM) 

6-Member's Mark (MM) 

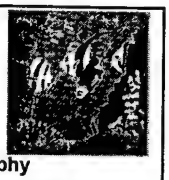
7-Sigma-Aldrich (SG) 

Hypothesis

- **Null hypothesis:**
  Fish oil supplements are consistent with one another in terms of their contents and chemical make-up; therefore, there is relatively no statistically significant difference between them in terms of their ability to promote apoptosis and inhibit cell adhesion of breast cancer cells.

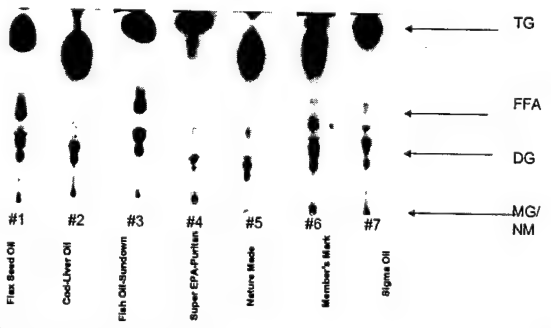


Methods



- **Analysis of Pure Lipid Composition**
  *** Techniques: Thin-Layer Chromatography**
- **Determination of Lipid Peroxidation/Omega-3 and Unsaturation Index**
  *** Techniques: Lipid Hydroperoxidation**
- **Identification of Total Fatty Acids**
  *** Techniques: Gas Chromatography**
- **Determination of anti-Breast Cancer Cell Metastasis**
  *** Techniques: Cell Adhesion**
- **Determination of Cytotoxic Effect on Breast Cancer Cells**
  *** Techniques: WST-1 Assay**

Lipid Composition of Supplements

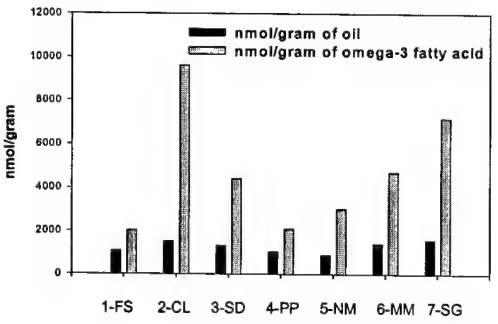


Lipid Peroxidation Results

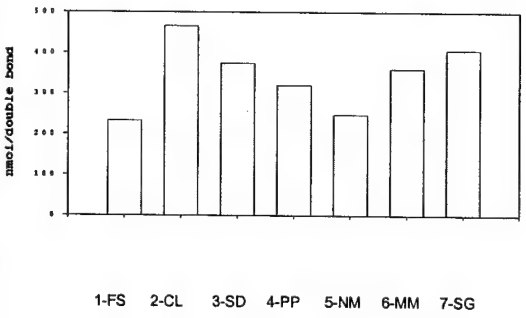
- Samples # 1 and 4 show the lowest lipid peroxidation/ omega-3 FA
- Samples # 5 and 6 contain Vitamin E yet do not show the lowest concentrations of oxidation (??)

Sample	Concentration(nmol) in 1 mL	Concentration of Omega 3/g sample	nmol/ g of oil	nmol/ g of w-3
1	1085.90	530 mg	1068	2015
2	1534.19	160 mg	1511	9624
3	1277.90	300 mg	1323	4410
4	951.95	500 mg	1045	2090
5	833.18	288 mg	876	3041
6	1359.16	300 mg	1415	4716
7	1505.61	220 mg	1584	7200

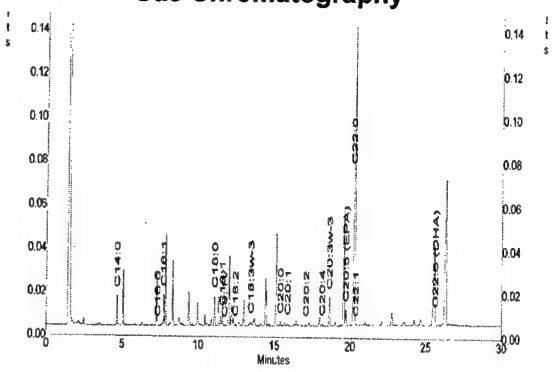
Lipid Peroxidation Results



Lipid Peroxidation- Unsaturation Index



Gas Chromatography



(#4- Puritan Pride Super EPA)

Gas Chromatography Results

(mg of Unsaturated FA / g of oil)

Sample #	14:1	16:1	18:1	18:2	18:3	20:1	20:4	20:5	22:6
1- Sundown Flax Oil	<1	<1	190	140	560	<1	<1	<1	<1
2- Sundown Cod- Liver Oil	<1	25	140	10	10	<1	20	130	130
3- Sundown Fish Oil	<1	300	90	20	10	3	5	140	100
4- Puritan's Pride	20	20	40	20	<1	<1	10	160	140
5- Nature Made	<1	10	110	80	5	5	10	170	110
6- Member's Mark	<1	50	80	20	2.0	10	10	190	130
7- Sigma	<1	4.0	100	30	100	20	10	160	150

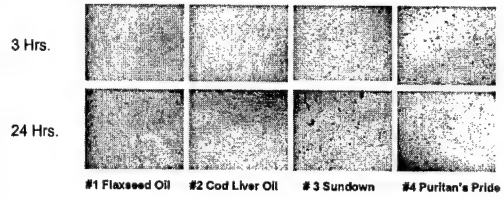
Biological Effects

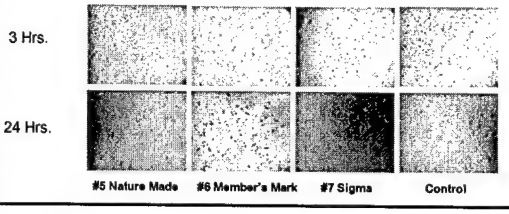
- **Metastasis- (Vitronectin Cell Adhesion Assay)**

  Incubated for 3 hour and 24 hour

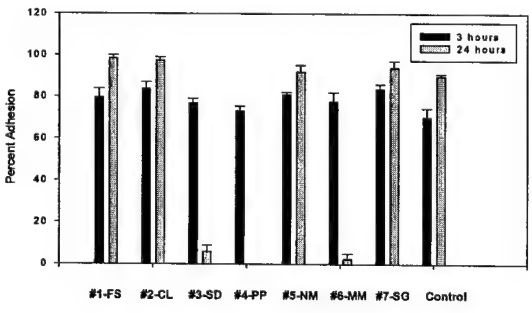
- **Cytotoxicity- (WST-1 Cell Viability Assay)**

  Incubated for 3 and 24 hours

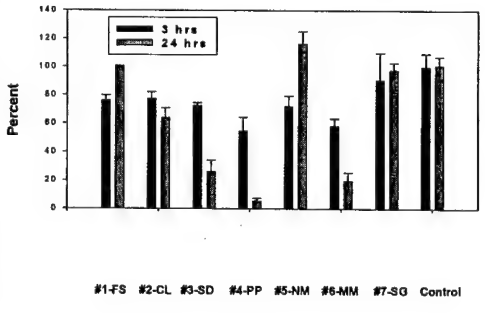




Vitronectin Adhesion Results



WST-1 Cell Viability Results



Conclusions

- Lipid Composition
  All samples are composed mainly of tri-,di-, and mono-glycerides with little amounts of free fatty acids in varying percentages
- Lipid Peroxidation
  Samples have varying degrees of lipid peroxidation/gram omega-3 FA.
- Gas Chromatography
  The majority of unsaturation in the samples arises from omega-3 content; other unsaturated fatty acids are contained in varying amounts not specified by the supplier
- Biological Effects
  The samples have varying effects on cell adhesion cell cytotoxicity

Conclusions

- Omega-3 supplements are not uniform
- It is difficult to determine the "best" brand
  (Our Results Suggest Puritan's Pride Super EPA)
- Retailers might want to consider collaborating so that overall capacity of the supplements are consistent

Acknowledgments

- **Rafat Siddiqui, PhD**
- **Gary Zaloga, MD**
- **Mustapha Zerouga, PhD**
- **Alicia Castillo**
- **Saame Raza Shaikh**

Role of Omega-3 Fatty Acids in Prevention of Cancer Induced Muscle Proteolysis

Heidi Yount
Rafat Siddiqui, PhD
Methodist Research Institute
August 8, 2003

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Background

- Muscle proteolysis, or cachexia, is characterized by a progressive loss of lean body mass
- Cachexia is common in cancer patients and estimated to be responsible for the death of approximately 22% of patients

Proteolytic Pathways

Muscle hypercatabolism is caused by an activation of a proteolytic pathway.

Three proteolytic pathways are believed to exist:

- Calpain (Calcium Dependent Neutral Protease)
- ATP-Ubiquitin
- Lysosomal

Muscle Proteolysis

Myofibrils

Desmin, Filmin, C-protein, Tropomyosin, Troponin T, Troponin I, Titlin, Nebulin, Vimentin, Gelsolin, Vinculin

↓ Calcium/Calpain dependent Mechanism

Myofilaments

Actin, MHC ↓ Ubiquitin/ Proteasome (Lysozymes)

Proteolysis

Hypothesis

Primary hypothesis:

- Tumor growth releases soluble proteolysis-inducing factor(s), which induce proteolysis by activating calpain enzymes.

Secondary hypothesis:

- Omega-3 fatty acids provide protection against the tumor-induced muscle wasting by inhibiting calpain activation.

In vivo Experiments

- Calpain Activity of heart and skeletal muscle tissue from nude mice implanted with MDA breast cancer cells
- Matrix Metalloproteinase (MMP) activity in tissue samples

Calpain Activity

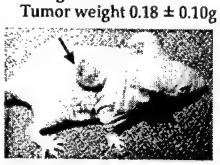
- MDA-MB-231 cells were implanted into nude mice
- For three weeks prior and three weeks post implantation the mice were fed a diet rich in ω-6 FA, ω-3 FA, or a balanced ω-3/ω-6 diet.
- Cardiac and skeletal muscle tissues were harvested and analyzed for calpain activity.

Diet Composition

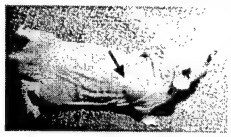
Ingredients (g/100g)	ω-6	Balanced	ω-3
Casein	20	20	20
Starch	25	25	25
Maltodextrin	5.0	5.0	5.0
Sucrose	30	30	30
D,L-Methionine	0.3	0.3	0.3
Mineral Mixture	3.5	3.5	3.5
Vitamin Mixture	1.0	1.0	1.0
Cellulose	5.0	5.0	5.0
Antioxidant	0.02	0.02	0.02
CholineBitartrate	0.2	0.2	0.2
Lipids:			
Corn Oil	**10**	**3.3**	**1.0**
Fish Oil	**—**	**6.7**	**9.0**
Ratio of ω-3/ω-6	**1/18**	**1/1**	**2.6/1**

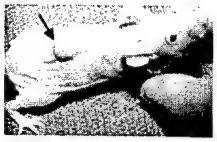
Mice

Omega-6 mouse
Tumor weight 0.18 ± 0.10g



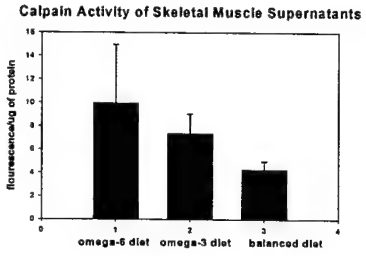
Omega-3 mouse
Tumor weight 0.13 ± 0.02g



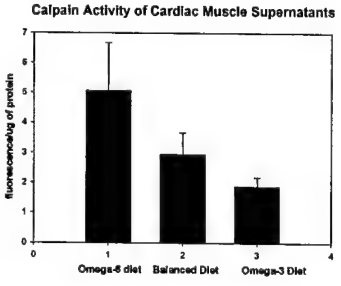


Balanced diet mouse
Tumor weight: 0.09 ± 0.01g

Calpain Results

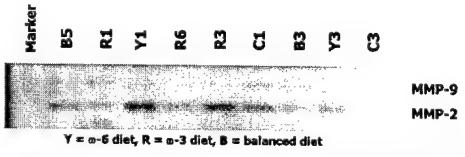


Calpain Results



Matrix Metalloproteinase Assay

- Matrix Metalloproteinase enzymes are involved in tumor invasion
- Assay uses Zymogram gels (acrylamide and gelatin) – the enzyme degrades gelatin, leaving light colored bands.



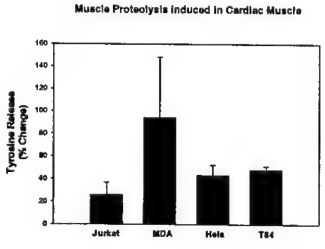
In vitro Experiments

- Induction of Proteolysis
- Inhibition of Proteolysis by DHA
- Identification of a Proteolysis Inducing Factor

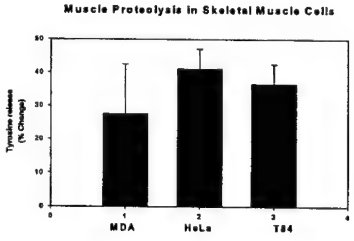
Induction of Proteolysis

- Cardiomyocytes and differentiated skeletal muscle cells were used to test the proteolytic effects of cancer cell extracts.
- Cancer cells used:
  - Jurkat (leukemia)
  - MDA-MB-231 (breast cancer)
  - HeLa (cervical cancer)
  - T84 (colon cancer)
- Proteolysis was evaluated through the release of ^{3}H-tyrosine

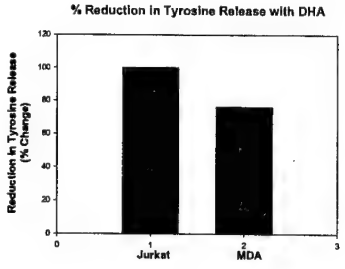
Results: Proteolysis Induced by Growth Medium



Results: Proteolysis Induced by Growth Medium



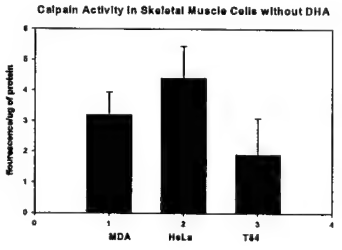
Inhibition by DHA



Calpain Activity of Skeletal Muscle Cells

- Skeletal muscle cells were plated and differentiated in a 96 well plate
- Cells were incubated overnight in the presence of DHA followed by overnight treatment with the cancer cell extracts.
- Calpain Activity was measured 60 minutes after the substrate was added.

Calpain Results

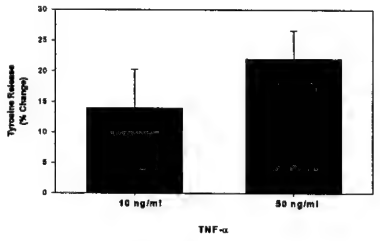


Identification of a Proteolysis Inducing Factor

Differentiated skeletal muscle cells were treated with factors believed to induce muscle proteolysis, including:

- Tumor Necrosis Factor-a
- C-reactive protein
- Lipopolysaccharide
- Interleukin-6
- Combinations of above

Proteolysis Induced by TNF



Further Experiments

- Proteolysis induced by the cancer cell growth media in cells treated with DHA
- MMP assays

Conclusion

In vivo

- Balanced diet or ω-3 diet reduced tumor weight in mice
- Calpain activation reduced by at least 50% in the balanced diet and ω-3 diet compared to the ω-6 diet

In vitro

- Cancer cell growth media for MDA, HeLa, and T84 cells induced proteolysis
- Calpain is activated in untreated skeletal muscle cells, and not activated in cells treated with DHA

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Mini-Reviews in Medicinal Chemistry, 2004, *4*, 859-871

Omega 3- Fatty Acids: Health Benefits and Cellular Mechanisms of Action

Rafat A. Siddiqui[1,2,3*], Saame R. Shaikh[3], Laura A. Sech[1], Heidi R. Yount[1], William Stillwell[3] and Gary P. Zaloga[1,2]

[1]*Methodist Research Institute, Clarian Health Partners, Indianapolis;* [2]*Department of Medicine, Indiana University Medical School, Indianapolis; and* [3]*Department of Biology, Indiana University-Purdue University, Indianapolis, Indiana, USA*

Abstract: Epidemiological evidence has established that ingestion of long chain, polyunsaturated omega-3 fatty acids, (ω-3 PUFAs) abundant in fish oils, have profound effects on many human disorders and diseases including cardiovascular disease and cancer. Here we briefly review the dietary recommendations and the food sources that are naturally enriched in these fatty acids. There are also a number of products including eggs, bread, and cereals available to supplement ω-3 fatty acid dietary intake. Some of these supplements are proposed to aid different pathological conditions. While the beneficial effects of omega-3 fatty acids can no longer be doubted, their molecular mechanism of action remains elusive. Without question, the action of omega-3 fatty acids is complex and involves a number of integrated signaling pathways. This review focuses on one of the possible cellular mechanisms by which the ω-3 PUFAs, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), may function. Studies with cancer cells suggest that DHA induces cell cycle arrest and apoptosis by activating protein phosphatases leading to dephosphorylation of retinoblastoma protein (pRB). Protein phosphatases are also involved with a protein Bcl2, which regulates the release of cytochrome c from mitochondria and eventually, activation of the apoptotic enzyme caspase 3.

Keywords: Omega 3- fatty acids, Cancer, Dietary intake, Food supplements.

INTRODUCTION

It is now evident that "all fats are not created equal". Some fats such as cholesterol saturated fats and polyunsaturated long chain omega-6 fatty acids taken in excess are considered "bad" for human health while the class of long chain polyunsaturated omega-3 fatty acids (ω-3 PUFAs) are beneficial. Table 1 presents a partial list of human afflictions that have been alleviated by ω-3 PUFAs. It is unclear how this class of very simple molecules can affect so many seemingly unrelated diseases. The reasons behind the beneficial properties of the omega-3 fatty acids are the subject of considerable interest and intense investigation.

EPIDEMIOLOGY STUDIES

The first clue that ω-3 PUFAs may exert beneficial effects on human health came from epidemiology studies on populations in which fish was a major component of the diet. The favorable health effects of ω-3 PUFAs on the cardiovascular system was initially recognized by Dyerberg *et al.* in the 1970s [1]. These researchers observed that Greenland Eskimos, who consumed a diet rich in ω-3s, had a low rate of cardiovascular disease as measured by a number of factors. Similar observations were also made for a Japanese fishing village that consumed an average of 250g of fish daily compared to a Japanese farming village that only averaged 90g of fish daily [2]. There are now many studies that have found an inverse association between fish oil consumption and risk of coronary heart disease (CHD) or sudden cardiac death in the general population [3-7]. In addition to the beneficial cardiovascular effects, use of fish oil was also reported to have anticancer properties. An epidemiology study of South African West Coast fisherman reported that despite smoking; high sodium intake; low consumption of fiber, fruits, and vegetables; absence of vitamin supplementation; and low levels of dietary micronutrients compared to urban whites, the fisherman had a lower incidence of colorectal cancer [8]. This was attributed to the protective effects of fish oil in their diets [8]. Similarly, a population-based case-control epidemiology study in Norway demonstrated an inverse relationship between serum ω-3 PUFA concentrations and thyroid cancer [9]. Cross-national studies have shown an inverse relationship between fish consumption and incidences of and mortality rates from prostate [10, 11] and breast cancer [12-16]. Furthermore, a series of case-controlled studies in Italy and Switzerland suggest that ω-3 PUFAs decrease the risk of several cancers, including oral and pharyngeal, esophageal, colon, breast, and ovarian cancers [17].

OMEGA-3 FATTY ACIDS AND THE DIET

Soon after the original epidemiology studies on fish oil diets became appreciated, it was evident that the "Western diet", rich in saturated fats, was partially responsible for the high incidence of cancer and heart disease associated with modern societies. An emphasis was then placed on substituting animal fats with unsaturated vegetable oils from corn, sunflower seeds, safflower seeds, cottonseed, and soybeans. Since these oils are rich in ω-6 fatty acids, there has been an associated increase in the ω-6/ω-3 dietary lipid ratio in Western societies. The ω-3 and ω-6 families of PUFAs function differently because of the location of the last double bond in the 3rd (omega-3) or 6th (omega-6)

*Address correspondence to this author at the Methodist Research Institute, 1800 N. Capital Avenue, oyes E504, Indianapolis, IN 46202, Tel: (317) 962-6941; Fax: (317) 962-9369; E-mail: rsiddiqu@clarian.org

1389-5575/04 $45.00+.00

Table 1. Reported Beneficial Effects of ω-3 PUFAs in Various Diseases

Disease/Disorders	Reference
ADHD	[135]
Aggression	[136]
Alcoholism	[137]
Arthritis	[138]
Asthma	[139,140]
Bipolar Disorder	[141]
Blindness	[142]
Cancer	[9,17,88,143-149]
Crohn's Disease	[150]
Cystic Fibrosis	[151]
Depression	[152]
Dermatitis	[153]
Dyslexia	[154]
Heart Disease	[155,156]
Hypersensitivity	[157]
Kidney Disease	[158]
Lupus Erythematosus	[159]
Malaria	[160]
Migraine Headaches	[161]
Multiple Sclerosis	[162]
Neurovisual Developmental Disorders	[163]
Nephropathy	[164]
Peroxisome Biogenesis Disorder	[165]
Phenylkenonuria	[166]
Psoriasis	[167]
Respiratory Diseases	[168]
Schizophrenia	[169]
Suicide	[152]
Ulcerative Colitis	[26,27]

positions from the methyl terminal of the aliphatic carbon chain Fig. (**1**). In a typical Western diet, the ratio of ω-6 to ω-3 fatty acids now ranges from approximately 20-30:1 instead of the range of 1-2:1, which is believed to have be present in the diets of prehistoric populations that survived on fresh fruits, vegetables, fish and animals [18]. A similar low ratio of ω-6/ω-3 dietary lipids has been reported for modern populations subsisting on a fish-based diet [1, 19]. Corresponding to this dramatic change in PUFA dietary ratio is an increased risk of cardiovascular, cancer, and other diseases among Western populations compared to populations that lived before the Industrial Revolution and those currently living on diets rich in fish oils [8, 9, 20-22]. The beneficial effects of fish oils are mostly attributed to their content of the ω-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

The 18-carbon ω-3 PUFA, α-linolenic acid, found in green leafy vegetables, flaxseed, rapeseed, and walnuts, can be desaturated and elongated in the human body to EPA and then to DHA. Therefore α–linolenic acid may have similar beneficial effects on health to the longer chain PUFAs. The ω-3 and ω-6 fatty acid families are important for human nutrition. The precursors of these fatty acids, 18-carbon linoleic (ω-6) and α-linolenic (ω-3), cannot be produced in the body and are therefore "essential" to the diet. Linoleic acid and linolenic acid are converted to longer chain ω-6 and ω-3 fatty acids by various cycles of desaturation and elongation as presented in Fig. (**2**).

The most common food sources of the long chain PUFA ω-3 fatty acids are cold-water fatty fish, including mackerel, salmon, herring, trout, sardines, and tuna (Table 2). Eggs and meat also contain small amounts of ω-3 fatty acids (Table 3). Increased intake of ω-3 PUFA can also occur through consumption of dietary α-linolenic acid, which can be metabolically converted, to EPA and DHA. Alpha-linolenic acid is a common component of flax seeds and canola oil. Flax seeds contain approximately 24% while canola oil contains approximately 11% α-linolenic acid. Canola oil is readily available in many foods such as bread and cereals, while energy bars often contain flax seed oils.

Because of the health benefits that ω-3 fatty acids provide, there is a need to set a Recommended Daily Allowance guideline for ω-3 fatty acids. As of yet, the United States Food and Drug Administration (FDA) has not made such a recommendation. In the typical Western diet, the average ω-3 fatty acid consumption is less than 0.1 grams/day. This is a very small amount considering that health authorities in Canada*, the United Kingdom†, and Australia‡ have made recommendations of 1-2 g ω-3 PUFAs/day. In 1999, as part of an effort to evaluate the importance of ω-3 PUFAs, a workshop was held at the National Institute of Health (Bethesda, Maryland, USA) to determine the recommended dietary intakes of ω-6 and ω-3 fatty acids. This workshop established an amount representing the Adequate Intake for Adults and Infants. For a 2000 kcal diet, the recommended intake of ω-3 fatty acids for adults was 0.65 grams/day, with a minimum intake of ω-3 fatty acids being 0.22 grams/day. The Adequate Intake for Infant Formula was set at approximately 2% of lipid intake [23]. More recently the American Heart Association recommended 1 gm/day of ω-3 PUFA for adults for the prevention of cardiovascular disease [24].

OMEGA-3 FATTY ACID SUPPLEMENTATION

Despite the fact that the Food and Drug Administration has yet to recommend precise dietary intake of ω-3 and ω-6

* Nutrition Recommendations. In S.R. Committee, Ed.; Minister of National Health and Welfare: Ottawa, ***1990***; pp. H49.

† Unsaturated fatty acids-nutritional and physiological significance: the report of the British Nutrition Foundation's Task Force. In C.a. Hall, Ed.; The British Nutrition Foundation: London, ***1992***.

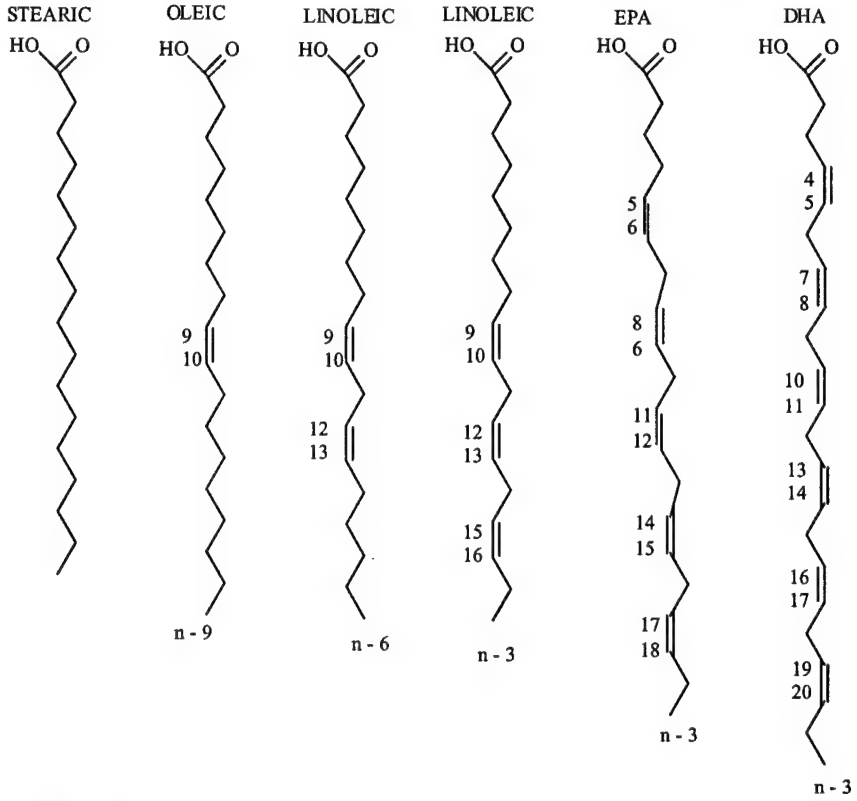

Fig. (1). Some biologically important fatty acids

Fatty acids are classified in saturated, ω-3, ω-6, or ω-9 families based on the position of the last double bond at the 3rd, 6th, or 9th position from the methyl terminal of the aliphatic carbon chain.

PUFAs, there is little doubt that fatty acids can have a profound effect on human health. This realization has paved the way for increased marketing of ω-3 supplements and patents for future drug development.

There are hundreds of web sites advertising the availability of ω-3 products as well as countless formulas rich in ω-3 that have been patented. Most of the patents contain different mixtures of ω-3 and ω-6 fatty acids and are suggested for the treatment of a myriad of disorders (Table 4). Although it is beyond the scope of this review to thoroughly discuss the countless applications of the patented formulas, a few representative examples follow. Their uses range from enhancing the weakened immune system of trauma patients to combating the effects of aging caused by cigarette smoke. Some formulations comprise a liquid drink while others may be administered enterally to ICU patients who have depressed absorption capacity. Other patents include a method for restoring gut integrity as well as a formulation to combat the negative symptoms found in postmenopausal women.

Perhaps the most abundant area for development of ω-3 patents has been in the treatment of inflammatory diseases. Compositions containing ω-3 esters have been developed and clinically proven to treat psoriasis and phlebitis [25] as well as ulcerative colitis [26, 27]. For the treatment of chronic inflammation as well as liver disorders, a combination of various ω-3 fatty acids has been developed in the form of medium-length triglycerides. The administration of ω-3 fatty acids as medium-length triglycerides speeds clearance of the lipid emulsions from the blood and therefore provides certain advantages. Enhanced blood clearance of these lipids results from stimulation of specific tissue uptake and inhibition of the synthesis of endogenous triglycerides. Thus, the uptake of ω-3s as medium-length triglycerides may also contribute to an overall reduction of blood triglycerides [28]. Furthermore, accompanying ω-3 PUFAs with medium-length triglycerides protects them from rapid oxidation, and this combination alone has a protective effect on the liver [29, 30].

Because of the increased research involving the health benefits of ω-3 fatty acids, several functional foods have been designed to enhance ω-3 PUFA intake (Table 5). Two functional foods have been designed for very specific purposes. For infants, Mead Johnson Nutritionals, (Bristol-Myers Pharmaceuticals, Evansville, IN) makes Enfamil Lipil™ brand baby formula, with levels of ω-3 PUFA comparable to breast milk and significantly higher than other commercial formulas. DHA in infants is important because it is essential for proper brain and eye development. For cancer patients, Ross Pharmaceutical (Abbott Laboratories, Columbus, OH) makes Prosure, a nutrition and energy beverage containing DHA designed to help people reverse tumor-induced weight loss. Omega-3 PUFAs intake may be increased by the use of ω-3 PUFAs enriched eggs,

‡ Report of the NHMRC working party: the role of polyunsaturated fats in the Australian diet. *1992.*

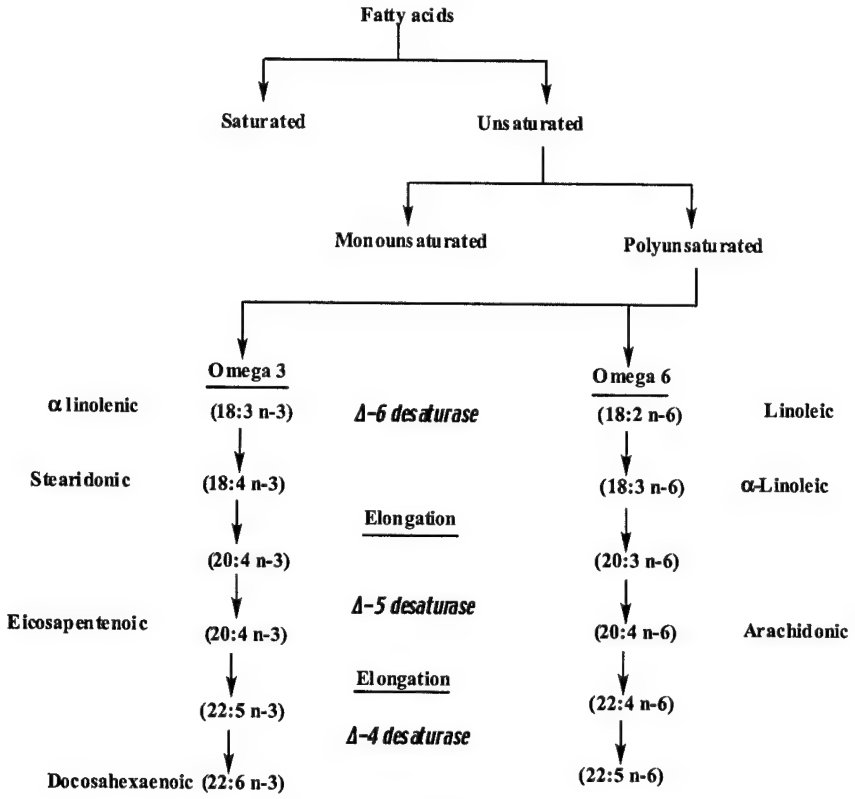

Fig. (2). Metabolic pathway of omega 6 and omega 3 fatty acid synthesis

Fatty acids are classified as saturated or unsaturated fatty acids depending on the presence of double bonds. The unsaturated fatty acids are further divided into monounsaturated or polyunsaturated fatty acids. The polyunsaturated fatty acids are either ω-3 or ω-6 fatty acids. α-linolenic acid and linoleic acid are the precursors of ω-3 and ω-6 fatty acids, respectively, and re converted to different long chain polyunsaturated fatty acid by sequential desaturation and elongation.

manufactured by many companies across Canada and the United States.

We analyzed seven different brands of ω-3 Fish oils (Sundown Flax Seed Oil, Sundown Cod Liver Oil, Sundown Fish Oil, Puritan's Pride Super EPA, Nature Made Fish Oil, Member's Mark Fish Oil, and Sigma-Aldrich Fish Oil) for their chemical composition (Table 6). It is clear from the labels of ingredients that different ω-3 supplements contain different amounts of ω-3 PUFA ranging from 160 to 550 mg/g of oil. Our gas chromatographic analysis data further indicate some differences in quantities of ω-3 PUFA in the capsules versus the amount that is marketed by the manufacturers. These discrepancies could be due to the fact that the oils are extracted from fish, which may be caught from different environmental conditions (cold water verses warm water) and in different seasons. These ω-3 PUFA supplements also have different levels of lipid peroxidation (data not shown) because each supplement may have been processed and stored differently as well as supplemented with different antioxidants. Furthermore, our data also indicate that fish oil supplements contain a significant quantity of other unsaturated fatty acids that nutrition labels neglect to reveal. While the appearance of other unsaturated fatty acids may have no effect on the ability of ω-3 PUFAs to function properly, the presence of other unsaturated fatty acids may have adverse biological effects. It is therefore important that these fatty acid supplements be evaluated and standarized against biological activites.

Because of the reputed ability of ω-3 fatty acids to support such a wide variety of health benefits, they could be considered as "wonder drugs". However, FDA does not classify these nutritional compounds as drugs and the FDA does not officially recognize them as treatments for the diseases. Despite this fact, the patented formulas and marketed nutritional supplements that contain ω-3 lipids will most certainly continue to be developed.

A POSSIBLE MODE OF ACTION FOR OMEGA-3 PUFA

Although epidemiological and nutritional evidence strongly suggest that ω-3 PUFAs have an influence on various disease states, the mechanism by which ω-3 PUFAs function remains unclear. Whatever omega-3 fatty acids mode of action is, it must be fundamental and commonly sheared by a wide variety of tissues. Several non-exclusive hypotheses regarding the mode of action of ω-3 PUFAs have been proposed. It has been suggested that ω-3 PUFAs may affect numerous membrane properties (e.g., permeability [31], "fluidity" [32], lipid packing [33], fusion [32], deformability [34] etc.); the activity of specific proteins (e.g., protein kinase C [35], rhodopsin [33], (Na+, K+)-

Table 2. ω-3 Fatty Acid (DHA and EPA) Content in Seafood[1]

Type	Amount of DHA (g/3oz portion)	Amount of EPA (g/3oz portion)	Amount Required to Provide ≈ 1 g of DHA and EPA
Catfish			
Farmed	0.116	0.085	15 oz
Wild	0.109	0.042	20oz
Clams	0.124	0.177	10 oz
Cod			
Atlantic	0.131	0.003	23 oz
Pacific	0.147	0.088	13 oz
Crab, Alaskan King	0.100	0.251	9 oz
Flounder/Sole	0.219	0.207	7 oz
Halibut	0.318	0.077	8 oz
Lobster	0.026	0.045	42 oz
Oyster			
Eastern	0.496	0.456	3 oz
Farmed	0.179	0.195	8 oz
Pacific	0.425	0.745	3 oz
Salmon			
Atlantic Farmed	1.238	0.587	2 oz
Atlantic Wild	1.215	0.349	2 oz
Pink Canned	0.685	0.718	2 oz
Scallops	0.092	0.076	18 oz
Shrimp	0.122	0.145	11 oz
Trout, Rainbow			
Farmed	0.697	0.284	3 oz
Wild	0.442	0.398	4 oz
Tuna			
Canned, light	0.190	0.040	13 oz
Fresh	0.970	0.309	2 oz
White	0.535	0.198	4 oz

[1]USDA Nutrient Data Laboratory. Http://www.nalusda.gov/fnic/foodcomp/. Accessed August 5, 2003.

ATPase [36], and Na+ channel [37]); lipid microdomain formation; [38-40] eicosanoid biosynthesis; [41] gene expression; [42] and formation of potent lipid peroxidation products [43]. It is likely that a combination of several of these effects are responsible for the beneficial health properties of ω-3 PUFAs. Here we will describe one possible molecular action of omega-3 fatty acids, their role in programmed cell death (apoptosis).

Table 3. Foods Naturally Containing ω-3 PUFAs[2]

Food	Amount of ω-3 PUFA (mg)
1 large hard-boiled egg	19
2 pieces fried chicken	37
3 oz tuna salad	47
12 large steamed shrimp	96
1 cup chicken livers	112
3 oz steamed crab	196
3 oz smoked salmon	227
3 oz beef liver	246
3 oz white tuna	535
3 oz salmon fillet	638

[2]U.S. Department of Agriculture, Agriculture Research Service, 1999. USDA Nutrient Database for Standard Reference, Release 13.

Omega-3 PUFAs induce apoptosis in cancer cells, whereas they protect neuronal, retinal, and cardiac cells against apoptosis. It is beyond the scope of this review to discuss the effect of ω-3s in every single health condition. Therefore, this review article will focus primarily on the role of ω-3 PUFAs in effecting signal transduction processes leading to apoptosis in various cancers.

Several reports have demonstrated that ω-3 PUFAs exert their anticancer effects on various cancer cell lines [44-47]. Dietary supplementation with ω-3 PUFAs (as a pure agent or in fish oil) increased apoptotic cell death in normal rat colonic cells [44-47], in transplantable rat Morris hepatocarcinoma 3924A [48] and Walker 256 carcinosarcoma [49]. Omega-3 PUFAs suppress the progression of human breast MDA-MB-231 [50, 51], MDA-MB-435 [52, 53], and KPL-1 cancer cells [54] in athymic nude mice. They increase survival time for dogs with lymphoma [55]; and reduce the

Table 4. Therapeutic Use of ω-3 PUFAs

Patent	Description	Inventor	Patent #
Glutamine-rich composition for immune system	For the purpose of treating patients whose immune systems have been weakened because of disease or trauma; the major ingredient is glutamine, which is accompanied by ω-3 and ω-6 PUFAs, arginine, and RNA.	Steven M. Ostrom	199861/10 USA
Formulation for menopausal women	For the purpose of providing nutritional supplementation for postmenopausal/menopausal women as well as relieving associated symptoms. The supplementation consists of various compounds including linoleic acid, linolenic acid, DHA, ω-2 fatty acids, and other ω-3 fatty acids.	Saul R. Levinson et al.	131236/10 USA
Composition for increase in ω-3 of human cell membranes	Proposes to increase the amount of ω-3 fatty acids that comprise cell membranes by way of a parenteral injection of fatty acid triglycerides in the form of an isotonic lipid emulsion.	Yvon A. Carpentier Isabelle E. Dupont	01117991 USA
Nutritional formula for ICU patients	Provides a formula for administration to intensive care patients who have weakened states of absorption capacity. The formula is given enterally and consists of a source of protein, carbohydrate, and ω-3/ω-6 fatty acids.	John Alexander et al.	96202637 USA
Formulation for smokers	Formula for combating the negative effects of smoking, such as aging of the skin; consists of a combination of ω-3 and ω-6 fatty acids.	David F. Horrobin	94301853 USA
Pharmaceutical composition for morbid affections	A composition containing esters of ω-3 PUFAs that have proven to be clinically useful in the treatment of psoriasis and phlebitis.	Tiberio Bruzzese et al.	93110903 USA
Formulation for protective effect on the liver	A combination of ω-3 fatty acids in their esterified form accompanied by medium-length triglycerides. The triglycerides are preferentially oxidized and thus protect the fatty acids from rapid oxidation. This combination helps to protect the liver and suppresses chronic inflammatory disorders.	Dr. Jorg Nehne Michael Boll	88116623 USA
Emulsion con-taining ω-3 fatty acids for treating inflam-matory diseases	An emulsion that contains multiple ω-3 fatty acids or their esters along with traditional additives for the treatment of ulcerative colitis.	Jeffrey Askanazi et al.	00637957/EP B1
Drinkable ω-3 preparation	A liquid nutritional drink consisting of ω-3 PUFAs in a water-based solution that does not turn rancid with time	Johan Myhre AS Coromar	00147377 WO
Composition for mainte-nance of gut integrity	A method for restoring gut integrity by adminis-tering a combination of ω-3 and ω-6 PUFAs.	Susan Marie Kaup	00035443 WO

risk of prostate cancer in humans [56]. Omega-3 PUFAs have also been shown to significantly reduce the incidence of tumor induction by dimethylbenz(a)anthracene in rats [57]. Addition of ω-3 PUFAs to the cultures of lung carcinoma A427 [58], Hep2 human larynx tumor cells [59], pancreatic Mia-Pa-Ca-2 cells [60], and embryonal carcinoma Tera-2 cells [61] induces apoptosis in these cell lines. Omega-3 PUFAs also inhibit the growth of cervical cells immortalized by the highly oncogenic human papillomavirus 16 (HPV16), foreskin keratinocytes immortalized by HPV16, and keratinocytes grown from papillomas with an HPV etiology [62]. Furthermore, conjugated DHA with a triene structure has been shown to induce apoptosis in DLD-1 cells (colorectal adenocarcinoma) without any effect on normal human fibroblast cell lines [63]. While there are many examples of ω-3 PUFAs-induced apoptosis, at present, the cellular and molecular mechanisms are unclear and a better understanding of the basic actions of ω-3 PUFAs will be needed before these polyunsaturated fatty acids can be fully employed in the clinic as anticancer agents [64, 65].

Many anticancer drugs exert their influence by inducing apoptosis. Apoptosis, or programmed cell death, is the physiological method by which unwanted or unneeded cells are eliminated during development or other biological processes [66]. It is also an important process in degenerative diseases, autoimmune disorders, and neoplasia development [67]. As a genetically regulated mechanism, apoptosis can occur through many pathways, but it is defined by several typical cellular and molecular events, including cell shrinkage, endoplasmic reticulum dilation, membrane blebbing, and extensive nuclear fragmentation [66]. Caspases, a family of cysteine proteases, play a critical role in apoptosis and are responsible for many of the biochemical and morphological changes associated with apoptosis [68-71].

OMEGA-3 FATTY ACIDS AND CYTOSOL-LINKED APOPTOSIS

Omega-3 PUFAs exert their anticancer effects by slowing down the growth of cancer cells via inhibition of cell cycle

Table 5. Functional Foods Containing ω-3 PUFA[3]

Product Type	Product	Manufacturer	ω-3 content/serving
Cereal	Healthy Scoop	Food by Design www.foodbydesign.com	2800 mg
Cereal	Cranberry Cereal Almonds Cereal Apple Cinnamon	Zoe Foods www.zoefoods.com	2400 mg
Cereal Bar	Healthy Break	Food by Design	2800 mg
Cereal Bar	Zoe Flax and Soy Bar -chocolate -peanut butter -apple crisp -lemon	Zoe Foods	1500 mg per bar for chocolate and peanut butter bars; 2200 mg per bar for apple crisp and lemon bars.
Cookies	Flax Macs	Food by Design	2400 mg
Eggs	Born 3 eggs	Born 3 Marketing Corp www.born3.com	400 mg
Mix	Flax Jacks (pancake and waffle mix)	Food by Design	5000 mg
Oil	Golden Omega-Omega oil	Naturalways www.naturalways.com/omega-omega	5750 mg

[3]Food websites found using www.flaxcouncil.ca/foodlist.

progression. However, in the continued presence of ω-3 PUFAs these arrested cells start dying thorough apoptosis. Previously, we demonstrated that DHA prolongs the S phase in cultured spleen lymphocytes [72]. Subsequently, other investigators demonstrated that ω-3 PUFAs arrest malignant cells in the S phase [73] and prevent G1/S progression in HT-29 human colonic cells [74], vascular smooth muscle cells [75], and urothelial cells [76]. These observations indicate that ω-3 PUFAs, particularly DHA, can exert their anticancer effects by arresting cell cycle progression. To date, however, little is known about the molecular and cellular events that lead to ω-3 PUFA-mediated cell cycle arrest and subsequent apoptosis. Progression through each phase of the cell cycle is tightly regulated and involves the expression and rapid degradation of the cyclin-dependent kinase (cdk) complex. In general, the levels of cdks are relatively constant throughout the cell cycle, while the cyclin levels vary substantially [77]. Cyclin A appears in the S phase with the onset of DNA synthesis [78]. Cyclin A associates initially with cyclin-dependent kinase-2 (cdk2) and later with cdk1 (also known as cell division control protein 2 or cdc2 and is involved in G2/M progression) [78]. This association, and hence the activities of cdk2 and cdc2, are essential for progression through the S phase to the G2 phase. Many of the effects of cyclin-cdks are mediated through phosphorylation of retinoblastoma protein (pRb) Fig. (**3**). pRb controls the progression of the cell cycle by regulating the activities of transcription factors, most importantly, E2F2 and E2F3. In a hypophosphorylated state, pRb physically associates with these transcriptional factors and blocks their ability to activate the gene expression of products necessary for cell cycle progression. Once phosphorylated, pRb loses much, if not all, of its growth inhibitory power and permits the advance into late G1, and hence, into the remainder of the cell cycle [79].

Table 6. Fatty Acid Composition of Selected ω–3 Supplements

	Monounsaturated	Omega 3 (mg/g oil)	Omega-6	w-3/w-6 ratios
Sundown Flax Oil	190 (150)	555 (530)	140 (129)	3.96
Sundown Cod- Liver Oil	165 (???)	266 (160)	21.3 (???)	12.48
Sundown Fish Oil	388 (???)	243 (300)	20 (???)	12.15
Puritan's Pride	73 (???)	527(500)	28(???)	10.51
Nature Made	123 (???)	286 (360)	91 (45)	3.16
Member's Mark	138 (???)	323 (300)	30 (???)	10.7
Sigma Chem. Co.	160 (120-260)	313 (180-30)	43 (<60)	7.28

Number in parenthesis indicates the manufacturer's reported values on the label.

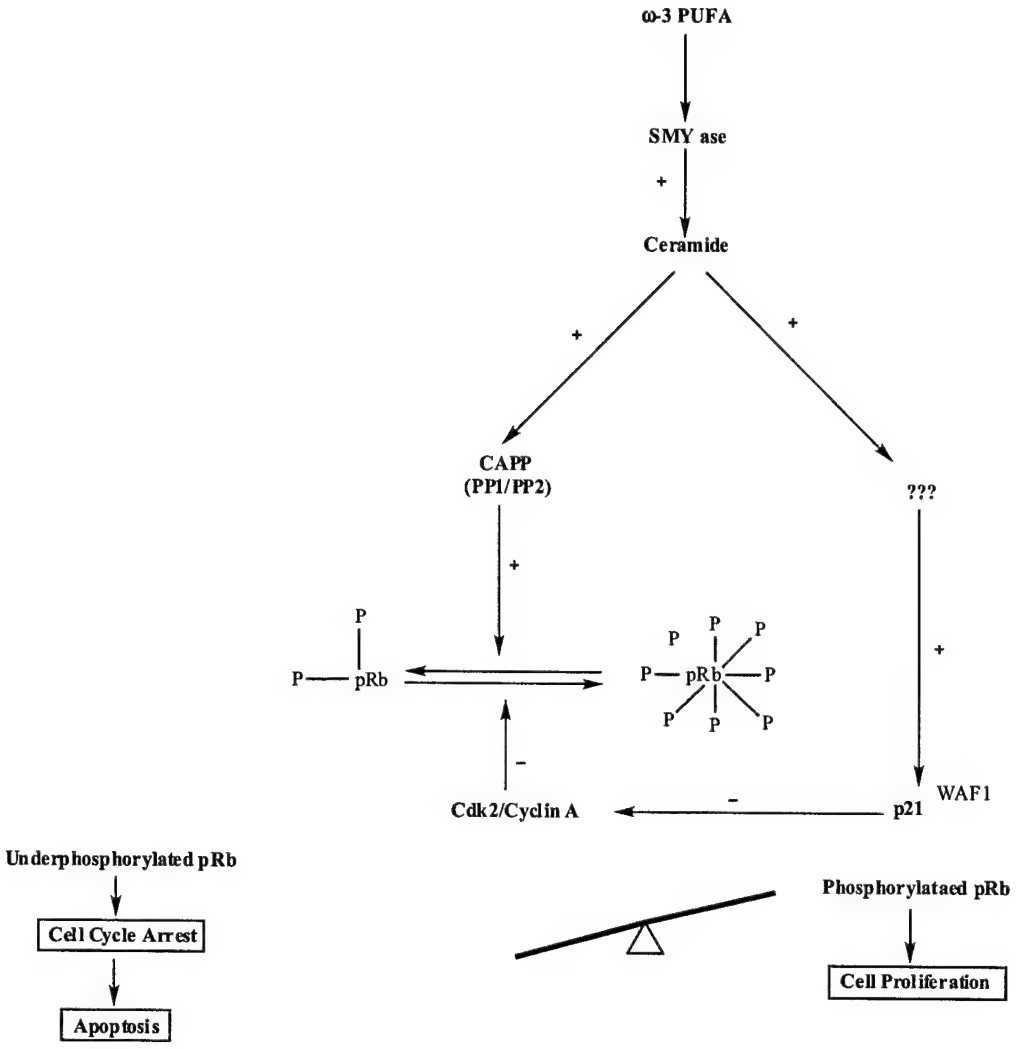

Fig. (3). Proposed action of ω-3 PUFAs on cell cycle arrest in cancer cells

Incorporation of ω-3 PUFAs into the cell membrane causes activation of sphingomyelinase (SMYase) and generation of ceramide. Ceramide mediates its effects via activation of ceramide-activated protein phosphatases (CAPP). Activation of CAPP (PP1 and/or PP2A) results in dephosphorylation of pRb phosphorylation. Ceramide also causes increased expression of $p21^{WAF1}$, and subsequently, inhibition of cyclin A/cdk2 activities. The overall effect of DHA results in hypophosphorylation of pRb protein and cell cycle arrest.

For the past 15 years our laboratory has investigated the relationship of DHA's alteration of membrane structure to its effects on cell signaling and apoptosis. Our initial studies used model lipid bilayers to explore the interaction of DHA with membrane phospholipids [80-85]. These experiments were then extended to Jurkat leukemia cells to elucidate the mechanism of the anticancer effects of DHA [86-88]. We demonstrated that low doses of DHA induce S-phase cell cycle arrest in Jurkat cells through hypophosphorylation of pRb by inhibiting cdk2 kinase activity and stimulating protein phosphatase activities [88]. Our earlier studies using a model lipid bilayer suggest that DHA incorporation into membranes containing sphingomyelin and cholesterol affects the formation of a type of lipid microdomain known as "lipid rafts" [39]. Lipid rafts may also contain sphingomyelinase (SMYase), an enzyme that generates ceramide, a potent second messenger involved in cell cycle arrest and apoptosis [89, 90]. Ceramide levels also change during progression of the cell cycle [91]. We therefore examined the levels of ceramide in DHA-induced growth-arrested cells. As predicted, our data demonstrate that DHA causes increased ceramide formation [92], probably resulting from DHA-induced activation of SMYase in the plasma membranes. Ceramide is a known potent activator of a protein phosphatase specific for cdk2 [92] and also functions as a modulator of pRb phosphorylation [91]. Furthermore, our research has demonstrated that DHA treatment of Jurkat cells leads to the activation of protein phosphatase 1 (PP1) and 2A (PP2A) [86, 87]. Therefore, it appears that DHA-induced ceramide formation leads to activation of protein phosphatases and then, subsequent to these events, dephosphorylation of pRb. Our studies also indicate that DHA induces elevated levels of $p21^{WAF1}$. Ceramide has been shown to enhance expression of p21 [92], a cellular inhibitor of cdk2 kinase. Through the p21 mechanism, it is possible that elevated levels of ceramide lead to inhibition of

cdk2 kinase. It therefore appears that DHA-induced ceramide may regulate phosphorylation of pRb by directly activating protein phosphatases and perhaps by inhibiting cyclin A/cdk-2 activities via increased expression of $p21^{WAF1}$. Although we have not yet studied the molecular mechanism by which ceramide leads to an increased expression of $p21^{WAF1}$, it is clear that PP1 is not an involved upstream of $p21^{WAF1}$ expression. A role for ceramide in the induction of p21 via activation of nuclear factor kappa-B (NFκB) and/or p53 has been established by various studies [93, 94]. A possible mechanism for DHA-induced cell cycle arrest is outlined in Fig. (**3**).

Furthermore, we observed that growth-arrested cells undergo apoptosis upon repeated treatment with low doses of DHA. This apoptosis process appears to be mediated via caspase-3 activation [88]. Previously we suggested that activation of caspase-3, and hence, induction of apoptosis by DHA, is also mediated through activation of protein phosphatases [86]. Our studies are consistent with several others that have shown that apoptosis can be mediated by activation of protein phosphatases. For example, Wolf and Eastman [95] demonstrated that activation of PP1 plays an important role in Fas-induced apoptosis by stimulating mitochondrial release of cytochrome C and caspase activation in HL-60 and Jurkat cells. Similarly, activation of a PP2A-like phosphatase has been demonstrated to play a key role in inducing apoptosis in a neuronal cell line [96]. Other studies have shown that CAPP, a member of the PP2A family, is involved in receptor-mediated induction of apoptosis in various cell lines [97]. These studies suggest that protein phosphatase activation may be a common feature of cells undergoing apoptosis (Fig. (**4**)). However, at present it is not clear how DHA activates protein phosphatases and how activation of protein phosphatases is linked to cell cycle arrest and induction of apoptosis. We did not test the role of

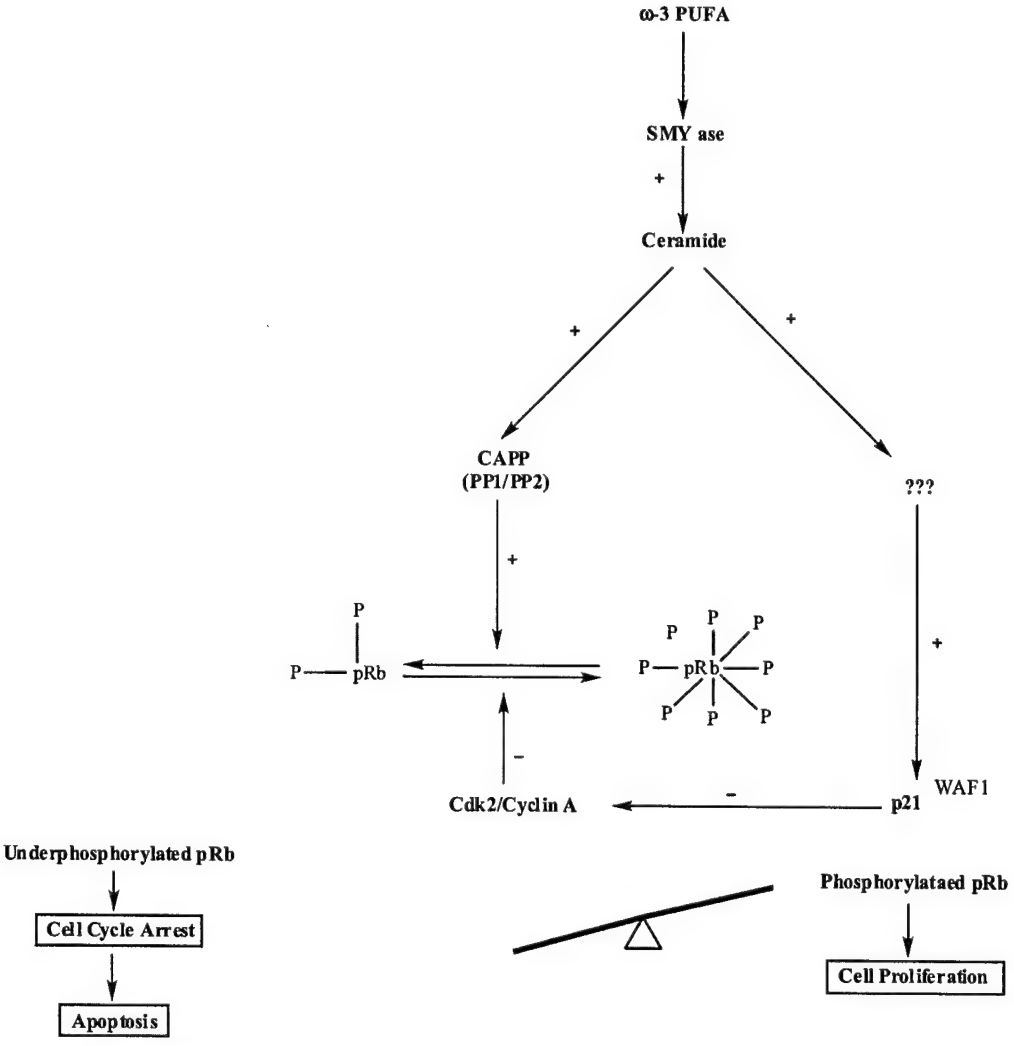

Fig. (4). Possible ω-3 PUFA-induced involvement of protein phosphatase in apoptosis. Activation of protein phosphates by ω-3 PUFA-induced ceramide formation can affect cancer cell growth through multiple pathways. Via dephosphorylation of retinoblastoma protein (pRB) protein phosphatases cause cell cycle arrest, which then leads to the induction of apoptosis. Protein phosphatases can also mediate release of cytochrome c and activation of caspases via dephosphorylation of Bcl2/Bid proteins. However, it is also possible that protein phosphatases play a direct role in the activation of caspase 3. Activation of the executionery caspase 3 then leads to the induction of apoptosis.

EPA in cell cycle arrest during this investigation. EPA can be converted to DHA and therefore, it can have effects similar to DHA on cell cycle arrest. Indeed several investigators have demonstrated that EPA also blocks cell cycle progression and induces apoptosis in Ramos cells [98], squamous cell carcinoma [99], vascular smooth muscle cells [75], HT29 colonic cells [100], and pancreatic PaCa-2 cancer cells [101]. In most cases the results are consistent with our finding of growth arrest in the S phase of cell cycle progression [75, 100, 101] through inhibition of cdk2 activities [75].

While our studies probed one possible pathway for DHA's effect on cell growth and viability, many other, often overlapping possible modes of action undoubtedly exist.

OMEGA-3 FATTY ACIDS AND MITOCHONDRIA-LINKED APOPTOSIS

The involvement of mitochondria in apoptosis has been demonstrated by several investigators in recent years [102-107] and this pathway has also been strongly implicated in ω-3 PUFA-induced cell death. Omega-3 PUFAs have been reported to alter mitochondrial membrane properties and functions in rat colonocytes [108], the human colon tumor cell line HT29 [109], Walker 256 rat carcinosarcoma [49], T24 [49], and Hep2 [59] cancer cells.

Evidence suggests that DHA, but not EPA, preferentially accumulates in cardiolipin [109]. Cardiolipin (CL) is a diphospholipid (diphosphatidylglycerol) required for mitochondrial structural integrity and for the proper function of the electron transport chain [110]. CL is absent from all other cell membranes other than mitochondria, where it is present in the inner membrane and at intermembrane contact sites [110]. In tissues with high respiration rates, such as heart, CL can account for 25% of the phospholipids in the inner-mitochondrial membrane [111], where it is usually bound to the enzyme complexes of electron transport and ATP synthesis (i.e. cytochrome *c* oxidase [112-114], NADH reductase [115, 116], cytochrome b_1c_1 complex [116, 117], and ATP synthase) [117, 118]. This suggests that mitochondrial function is very much dependent on the proper amount of CL. The CL acyl composition is sensitive to diet, and in humans it is usually rich in the essential dietary fatty acid linoleic acid (LA, 18:2 n-6) [119]. However, any change in dietary fatty acids is reflected in a change in acyl composition of CL. In mammals, CL has been modified to contain 85-90 mol% LA [120, 121], 50 mol% DHA [122], or 50 mol% oleic acid (OA) [122]. It is believed that ω-3 PUFAs in CL are susceptible to reactive oxygen species (ROS), which are generated through oxidative phosphorylation. CL is peroxidized by ROS and this process results in a decrease in CL levels in the mitochondrial membrane [123]. It has been suggested that low levels of CL either by peroxidation or its decreased synthesis [124, 125] compromises the integrity of CL-dependent proteins involved in energy metabolism, causing a drop in mitochondrial membrane potential, which in turn initiates apoptosis [126].

Consistent with these suggestions, cancer cells treated with ω-3 fatty acids clearly exhibit alterations in mitochondrial membrane potential and undergo apoptosis [49, 59, 108, 109]. Recently, a number of studies have reported a mechanism of mitochondrial-mediated apoptosis. It has been suggested that the peroxidation and/or loss of CL induces cytochrome C release from mitochondria. Studies have shown a highly significant temporal correlation of CL depletion with cytochrome C release to the cytosol [110]. The integrity of mitochondria and release of cytochrome C are regulated by Bcl-2 family members residing in the outer mitochondrial membrane [127]. Bcl-2 family members encode proteins that can be either antiapoptotic (e.g., Bcl-2, Bcl-X_L) or pro-apoptotic (e.g., Bax, Bcl-X_S, Bak, Bad, Bid) and therefore integrate signals from growth and death stimuli. An excess of Bcl-2 antiapoptotic proteins over Bcl-2 proapoptotic proteins protects the integrity of mitochondria and prevents cytochrome C release, whereas an excess of proapoptotic Bcl-2 proteins over antiapoptotic Bcl-2 proteins allows leakage of cytochrome C. It has been demonstrated recently that one chain of CL is inserted into a hydrophobic channel in cytochrome C, whereas another acyl chain extends into the bilayer [128]. CL is released from mitochondria for degradation in peroxisomes [129]. The proapoptotic protein Bid plays a role in the transfer of CL [130]. It has been shown that CL transfer occurs at the same concentrations of Bid that lead to mitochondrial release of cytochrome C [130]. The activities of the Bcl-2 family are regulated by different mechanisms, such as homo- and heterodimerization with other family members and also by proteolysis and phosphorylation [131]. There are recent reports that antiapoptotic Bcl-2 is inactivated by phosphatases, particularly by PP2A [132]. We have demonstrated that DHA treatment of Jurkat leukemia cells results in ceramide formation [88], which is also known to activate a phosphatase with characterised PP2A-type properties [97]. Similarly, dephosphorylation of Bad (proapoptotic) results in its activation and binding to Bcl-2, initiating cytochrome C release. The release of cytochrome C then interacts with Apaf-1 and dATP, leading to caspase 9 activation and hence downstream execution of the caspase cascade [133, 134]. The effector caspases are active proteases that then lead to morphological changes characteristic of apoptotic cell death, such as membrane blebbing and formation of apoptotic vesicles, cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation. The omega-3-induced steps in mitochondria-linked apoptosis are outlined in Fig. (**4**).

SUMMARY

Epidemiological and dietary studies strongly indicate that ω-3 PUFAs provide tremendous health benefits for a number of diseases including cancer and heart disease. ω-3 PUFAs are readily obtained from naturally occurring foods, manufactured functional foods, or from ω-3 PUFA supplements. One has to be careful, however, as our study suggests that different brands of supplements contain widely different amounts of ω-3 PUFAs and other mono and polyunsaturated fatty acids and cholesterol. ω-3 PUFAs have been shown to alter biologically essential processes including cell signaling and apoptosis. Here we reviewed how ω-3 PUFAs might regulate cellular signaling pathways and *induce* apoptosis through cytosolic and mitochondrial mediated signaling pathways. But even this is

controversial, as ω-3 PUFAs have been shown to *prevent* apoptosis in heart, neuronal, and retinal tissues. In these organs, ω-3 PUFAs appear to preserve function and exhibit anti-apoptotic properties through similar cellular signaling pathways that induce apoptosis in other organs. Comparing details of the effects of ω-3 PUFAs on cell signaling in different tissues therefore offers a unique approach in developing ω-3 PUFA-containing drugs. These drugs may selectively destroy cancer cells while preserving the vital physiological functions of other healthy tissues.

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REFERENCES

[1] Dyerberg, J.; Bang, H.O.; Hjorne, N. *Am. J. Clin. Nutr.*, **1975**, *28*, 958.
[2] Hirai, A.; Terano, R.; Saito, H.; Tamura, Y.; Yoshida, S. In *Nutritional Prevention of Cardiovascular Diseases*; W. Levenburg and Y. Yamori, Ed.; Academic Press: New York, **1984**; pp. 231.
[3] Kromhout, D.; Bosschieter, E.B.; de Lezenne Coulander, C. *N. Engl. J. Med.*, **1985**, *312*, 1205.
[4] Daviglus, M.; Stamler, J.; Orencia, A.J.; Dyer A.R.; Liu, K.; Greenland, P.; Walsh, M.K.; Morris, D.; Shekelle, R.B. *N. Engl. J. Med.*, **1997**, *336*, 1046.
[5] Albert, C.; Hennekens, C.H.; O'Donnell, C.J.; Ajani, U.A.; Carey, V.J.; Willett, W.C.; Ruskin, J.N.; Manson, J.E. *JAMA*, **1998**, *279*, 23.
[6] Hu, F.; Bronner, L.; Willett, W.C.; Stampfer, M.J.; Rexrode, K.M.; Albert, C.M.; Hunter, D.; Manson, J.E. *JAMA*, **2002**, *287*, 1815.
[7] Albert, C.; Campos, H.; Stampfer, M.J.; Ridker, P.M.; Manson, J.E.; Willett, W.C.; Ma, J. *N. Engl. J. Med.*, **2002**, *346*, 1113.
[8] Schloss, I.; Kidd, M.S.; Tichelaar, H.Y.; Young, G.O.; O'Keefe, S.J. *S. Afr. Med. J.*, **1997**, *87*, 152.
[9] Berg, J.P.; Glattre, E.; Haldorsen, T.; Hostmark, A.T.; Bay, I.G.; Johansen, A.F.; Jellum, E. *Cancer Causes Control*, **1994**, *5*, 433.
[10] Hebert, J.R.; Hurley, T.G.; Olendzki, B.C.; Teas, J.; Ma, Y.; Hampl, J.S. *J. Natl. Cancer Inst.*, **1998**, *90*, 1637.
[11] Kobayashi, M.; Sasaki, S.; Hamada, G.S.; Tsugane, S. *Japn. J. Cancer Res.*, **1999**, *90*, 914.
[12] Hebert, J.R.; Rosen, A. *Cancer Detect Prev.*, **1996**, *20*, 234.
[13] Kaizer, L.; Boyd, N.F.; Kriukov, V.; Tritchler, D. *Nutr. Cancer*, **1989**, *12*, 61.
[14] Sasaki, S.; Horacsek, M.; Kesteloot, H. *Prev. Med.*, **1993**, *22*, 187.
[15] Guo, W.D.; Chow, W.H.; Zheng, W.; Li, J.Y.; Blot, W.J. *Jpn. J. Cancer Res.*, **1994**, *85*, 572.
[16] Caygill, C.P.; Charlett, A.; Hill, M.J. *Br. J. Cancer*, **1996**, *74*, 159.
[17] Tavani, A.; Pelucchi, C.; Parpinel, M.; Negri, E.; Franceschi, S.; Levi, F.; La Vecchia, C. *Int. J. Cancer*, **2003**, *105*, 113.
[18] Eaton, S.B.; Eaton, S.B., 3rd; Sinclair, A.J.; Cordain, L.; Mann, N.J. *World Review of Nutrition & Dietetics*, **1998**, *83*, 12.
[19] Bang, H.O.; Dyerberg, J.; Sinclair, H.M. *Amer. J. Clin. Nutr.*, **1980**, *33*, 2657.
[20] Norell, S.E.; Ahlbom, A.; Feychting, M.; Pedersen, N.L. *Br. Medi. J. Clin. Res. Ed.*, **1986**, *293*, 426.
[21] Shekelle, R., Missell, LV, Paul, O, Shyrock, AM, Stamler, J. *N. Engl. J. Med.*, **1985**, *313*, 820.
[22] Dolecek, T., Grandits, G. World Review of Nutrition & Dietetics, **1991**, 66, 205.
[23] Simopoulos, A.P.; Leaf, A.; Salem, N., Jr. *J. Amer. Coll. Nutr.*, **1999**, *18*, 487.
[24] Kris-Etherton, P.M.; Harris, W.S.; Appel, L.J. *Circulation*, **2002**, *106*, 2747.
[25] Mayser, P.; Mrowietz, U.; Arenberger, P.; Bartak, P.; Buchvald, J.; Christophers, E.; Jablonska, S.; Salmhofer, W.; Schill, W.B.; Kramer, H.J.; Schlotzer, E.; Mayer, K.; Seeger, W.; Grimminger, F. *J. Am. Acad. Dermatology*, 1998, *38*, 539.
[26] Nieto, N.; Torres, M.I.; Rios, A.; Gil, A. *J. Nutr.*, **2002**, *132*, 11.
[27] Aslan, A.; Triadafilopoulos, G. *Amer. J. Gastroenterology*, **1992**, *87*, 432.
[28] Qi, K.; Seo, T.; Al-Haideri, M.; Worgall, T.S.; Vogel, T.; Carpentier, Y.A.; Deckelbaum, R.J. *Biochemistry*, **2002**, *41*, 3119.
[29] Chan, S.; McCowen, K.C.; Bistrian, B. *Curr. Opin. Clin. Nutr. Metab. Care*, **1998**, *1*, 163.
[30] Yeh, S.L.; Chang, K.Y.; Huang, P.C.; chen, W.J. *Nutrition*, **1997**, *13*, 32.
[31] Stillwell, W.; Jenski, L.J.; Crump, F.T.; Ehringer, W. *Lipids*, **1997**, *32*, 497.
[32] Ehringer, W.; Belcher, D.; Wassall, S.R.; Stillwell, W. *Chem. Phys. Lipids*, **1990**, *54*, 79.
[33] Litman, B.J.; Mitchell, D.C. *Lipids*, **1996**, *31 Suppl*, S193.
[34] Zerouga, M.; Jenski, L.J.; Booster, S.; Stillwell, W. *Cancer Lett.*, **1997**, *119*, 163.
[35] Slater, S.J.; Kelly, M.B.; Taddeo, F.J.; Ho, C.; Rubin, E.; Stubbs, C.D. *J. Biol. Chem.*, **1994**, *269*, 4866.
[36] Huang, W.H.; Kakar, S.S.; Askari, A. *Biochem. Int.*, **1986**, *12*, 521.
[37] Kang, J.X.; Leaf, A. Proc. Natl. Acad. Sci. U. S. A., **1996**, 93, 3542.
[38] Clamp, A.G.; Ladha, S.; Clark, D.C.; Grimble, R.F.; Lund, E.K. *Lipids*, **1997**, *32*, 179.
[39] Shaikh, S.R., Dumaual, A.C., Jenski, L.J., Stillwell, W. *Biochim. Biophys. Acta*, **2001**, *1512*, 317.
[40] Shaikh, S.R.; Brzustowicz, M.R.; Gustafson, N.; Stillwell, W.; Wassall, S.R. *Biochem. Int.*, **2002**, *41*, 10593.
[41] Urquhart, P.; Parkin, S.M.; Nicolaou, A. *Prostaglandins Leukot. Essent. Fatty Acids*, **2001**, *65*, 15.
[42] Jump, D.B.; Clarke, S.D. *Annu. Rev. Nutr.*, **1999**, *19*, 63.
[43] Narayanan, B.A.; Narayanan, N.K.; Reddy, B.S. *Int. J. Oncol.*, **2001**, *19*, 1255.
[44] Hatala, M.A.; Rayburn, J.; Rose, D.P. *Lipids*, **1994**, *29*, 831.
[45] Beck, S.A.; Smith, K.L.; Tisdale, M.J. *Cancer Res.*,**1991**,*51*, 6089.
[46] Rose, D.P.; Connolly, J.M. *Cancer Res.*, **1990**, *50*, 7139.
[47] Madhavi, N.; Das, U.N. *Cancer Lett.*, **1994**, *84*, 31.
[48] Calviello, G.; Palozza, P.; Piccioni, E.; Maggiano, N.; Frattucci, A.; Franceschelli, P.; Bartoli, G.M. *Int. J. Cancer*, **1998**, *75*, 699.
[49] Colquhoun, A.; Schumacher, R.I. *Biochim. Biophys. Acta*, **2001**, *1533*, 207.
[50] Connolly, J.M.; Gilhooly, E.M.; Rose, D.P. *Nutr. Cancer*, **1999**, *35*, 44.
[51] Rose, D.P.; Connolly, J.M. *Int. J. Oncol.*, **1999**, *15*, 1011.
[52] Liu, Y.; Liu, X.H. *Cancer Lett.*, **1995**, *92*, 209.
[53] Rose, D.P.; Connolly, J.M.; Rayburn, J.; Coleman, M. *J. Natl. Cancer Inst.*, **1995**, *87*, 587.
[54] Senzaki, H., Iwamoto, S., Ogura, E., Kiyozuka, Y., Arita, S., Kurebayashi, J., Takada, H., Hioki, K., Tsubura, A. *Anticancer Res.*, **1998**, *18*, 1621.
[55] Ogilvie, G.K.; Fettman, M.J.; Mallinckrodt, C.H.; Walton, J.A.; Hansen, R.A.; Davenport, D.J.; Gross, K.L.; Richardson, K.L.; Rogers, Q.; Hand, M.S. *Cancer*, **2000**, *88*, 1916.
[56] Norrish, A.E.; Skeaff, C.M.; Arribas, G.L.; Sharpe, S.J.; Jackson, R.T. *Br. J. Cancer*, **1999**, *81*, 1238.
[57] Noguchi, M.; Minami, M.; Yagasaki, R.; Kinoshita, K.; Earashi, M.; Kitagawa, H.; Taniya, T.; Miyazaki, I. *Br. J. Cancer*, **1997**, *75*, 348.
[58] Rudea, P.K.; Krokan, H.E. *Anticancer Res.*, **1999**, *19*, 461.
[59] Colquhoun, A. Biochem. Mol. Biol. Int., **1998**, 45, 331.
[60] Hawkins, R.A.; Sangster, K.; Arends, M.J. *J. Pathol.*, **1998**, *185*, 61.
[61] Timmer-Bosscha, H.; de Vries, E.G.; Meijer, C.; Oosterhuis, J.W.; Mulder, N.H. *Cancer Chemother. Pharmacol.*, **1998**, *41*, 469.
[62] Chen, D.; Auborn, K. *Carcinogenesis*, **1999**, *20*, 249.
[63] Igarashi, M.; Miyazawa, T. *Biochem. Biophys. Res. Commun.*, **2000**, *270*, 649.
[64] Hawkes, N. *Br. Med. J.*, **1994**, *309*, 543.
[65] Wigmore, S.J.; Ross, J.A.; Falconer, J.S.; Plester, C.E.; Tisdale, M.J.; Carter, D.C.; Fearon, K.C. *Nutrition*, **1996**, *12*, S27.
[66] Cohen, G.M.; Sun, X.M.; Snowden, R.T.; Dinsdale, D.; Skilleter, D.N. *Biochem. J.*, **1997**, *286*, 331.
[67] Cohen, J.J.; Duke, R.C.; Fadok, V.A.; Sellins, K.S. *Annu. Rev. Immunol.*, **1992**, *10*, 267.
[68] Wyllie, A.H.; Kerr, J.F.; Currie, A.R. *Int. Rev. Cytol.*, **1980**, *68*, 251.
[69] Kumar, S. Trends Biochem. Sci., **1995**, 20, 198.

[70] Yuan, J.; Shaham, S.; Ledoux, S.; Ellis, H.M.; Horvitz, H.R. *Cell*, **1993**, *75*, 641.

[71] Sun, X.M.; MacFarlane, M.; Zhuang, J.; Wolf, B.B.; Green, D.R.; Cohen, G.M. *J. Biol. Chem.*, **1999**, *274*, 5053.

[72] Scherer, J.M.; Stillwell, W.; Jenski, L.J. *Cell Immunol.*, **1997**, *180*, 153.

[73] Albino, A.P.; Juan, G.; Traganos, F.; Reinhart, L.; Connolly, J.; Rose, D.P.; Darzynkiewicz, Z. *Cancer Res.*, **2000**, *60*, 4139.

[74] Chen, Z.Y.; Istfan, N.W. Prostaglandins Leukot. Essent. Fatty Acids, **2001**, 64, 67.

[75] Terano, T.; Tanaka, T.; Tamura, Y.; Kitagawa, M.; Higashi, H.; Saito, Y.; Hirai, A. *Biochem. Biophys. Res. Commun.*, **1999**, *254*, 502.

[76] Diggle, C.P.; Pitt, E.; Roberts, P.; Trejdosiewicz, L.K.; Southgate, J.; Diggle, L. *J. Lipid Res.*, **2000**, *41*, 1509.

[77] Lundberg, A.S.; Weinberg, R.A. *Eur. J. Cancer*, **1999**, *35*, 531.

[78] Stillman, B. *Science*, **1996**, *274*, 1659.

[79] Mittnacht, S. Curr. Opin. Genet. Dev., **1998**, 8, 21.

[80] Schofield, M.; Jenski, L.J.; Dumaual, A.C.; Stillwell, W. *Chem. Phys. Lipids*, **1998**, *95*, 23.

[81] Williams, E.E.; Jenski, L.J.; Stillwell, W. *Biochim. Biophys. Acta*, **1998**, *1371*, 351.

[82] Kafrawy, O.; Zerouga, M.; Stillwell, W.; Jenski, L.J. *Cancer Lett.*, **1998**, *132*, 23.

[83] Williams, E.E.; May, B.D.; Stillwell, W.; Jenski, L.J. *Biochim. Biophys. Acta*, **1999**, *1418*, 185.

[84] Stillwell, W.; Jenski, L.J.; Zerouga, M.; Dumaual, A.C. *Chem. Phys. Lipids*, **2000**, *104*, 113.

[85] Williams, E.E.; Cooper, J.A.; Stillwell, W.; Jenski, L.J. *Mol. Membr. Biol.*, **2000**, *17*, 157.

[86] Siddiqui, R.A., Jenski LJ, Neff K, Harvey K, Kovacs R, Stillwell W. *Biochim. Biophys. Acta*, **2001**, *1499*, 265.

[87] Siddiqui, R.A.; Jenski, L.J.; Wiesehan, J.D.; Hunter, M.V.; Kovacs, R.J.; Stillwell, W. *Biochim. Biophys. Acta*, **2001**, *1541*, 188.

[88] Siddiqui, R.; Jenski, L.; Harvey, K.; Wiesehan, J.D.; Stillwell, W.; Zaloga, G.P. *Biochem. J.*, **2003**, *371*, 621.

[89] Tonnetti, L.; Veri, M.C.; Bonvini, E.; D'Adamio, L. *J. Exp. Med.*, **1999**, *189*, 1581.

[90] Jayadev, S.; Liu, B.; Bielawska, A.E.; Lee, J.Y.; Nazaire, F.; Pushkareva, M.; Obeid, L.M.; Hannun, Y.A. *J. Biol. Chem.*, **1995**, *270*, 2047.

[91] Lee, J.Y.; Leonhardt, L.G.; Obeid, L.M. *Biochem. J.*, **1998**, *334*, 457.

[92] Lee, J.Y.; Bielawska, A.E.; Obeid, L.M. *Exp. Cell Res.*, **2000**, *261*, 303.

[93] Kim, W.H.; Kang, K.H.; Kim, M.Y.; Choi, K.H. *Biochem. Cell Biology*, **2000**, *78*, 127.

[94] Hellin, A.C.; Bentires-Alj, M.; Verlaet, M.; Benoit, V.; Gielen, J.; Bours, V.; Merville, M.P. *J. Pharm. & Exper. Therap.*, **2000**, *295*, 870.

[95] Wolf, C.M.; Eastman, A. *Exp. Cell Res.*, **1999**, *247*, 505.

[96] Mills, J.C.; Lee, V.M.; Pittman, R.N. *J. Cell Sci.*, **1998**, *111*, 625.

[97] Chalfant, C.E.; Kishikawa, K.; Mumby, M.C.; Kamibayashi, C.; Bielawska, A.; Hannun, Y.A. *J. Biol. Chem.*, **1999**, *274*, 20313.

[98] Heimli, H.; Giske, C.; Naderi, S.; Drevon, C.A.; Hollung, K. *Lipids*, **2002**, *37*, 797.

[99] Palakurthi, S.S.; Fluckiger, R.; Aktas, H.; Changolkar, A.K.; Shahsafaei, A.; Harneit, S.; Kilic, E.; Halperin, J.A. *Cancer Res.*, **2000**, *60*, 2919.

[100] Clarke, R.G.; Lund, E.K.; Latham, P.; Pinder, A.C.; Johnson, I.T. *Lipids*, **1999**, *34*, 1287.

[101] Lai, P.B.; Ross, J.A.; Fearon, K.C.; Anderson, J.D.; Carter, D.C. *Br. J. Cancer*, **1996**, *74*, 1375.

[102] Kroemer, G. Biochem. Biophys. Res. Commun., **2003**, 304, 433.

[103] Newmeyer, D.D.; Ferguson-Miller, S. *Cell*, **2003**, *112*, 481.

[104] Castedo, M.; Ferri, K.; Roumier, T.; Metivier, D.; Zamzami, N.; Kroemer, G. *J. Immunol. Methods*, **2002**, *265*, 39.

[105] Roberts, D.L.; Goping, I.S.; Bleackley, R.C. *Biochem. Biophys. Res. Commun.*, **2003**, *304*, 513.

[106] Parcellier, A.; Gurbuxani, S.; Schmitt, E.; Solary, E.; Garrido, C. *Biochem. Biophys. Res. Commun.*, **2003**, *304*, 505.

[107] van Gurp, M.; Festjens, N.; van Loo, G.; Saelens, X.; Vandenabeele, P. *Biochem. Biophys. Res. Commun.*, **2003**, *304*, 487.

[108] Chapkin, R.S.; Hong, M.Y.; Fan, Y.Y.; Davidson, L.A.; Sanders, L.M.; Henderson, C.E.; Barhoumi, R.; Burghardt, R.C.; Turner, N.D.; Lupton, J.R. *Lipids*, **2002**, *37*, 193.

[109] Watkins, S.M.; Carter, L.C.; J.B., G. *J. Lipid Res.*, **1998**, *39*, 1583.

[110] McMillin, J.B.; Dowhan, W. *Biochim. Biophys. Acta*, **2002**, *1585*, 97.

111] Daum, G. Biochim. Biophys. Acta, **1985**, 822, 1.

[112] Robinson, N.C.; Zborowski, J.; Talbert, L.H. *Biochem.*, **1990**, *29*, 8962.

[113] Vik, S.B.; Georgevich, G.; Capaldi, R.A. *Proc. Natl. Acad. Sci. U. S. A.*, **1981**, *78*, 1456.

[114] Robinson, N.C. J. Bioenerg. Biomembr., **1993**, 25, 153.

[115] Fry, M.; Green, D.E. Biochem. Biophys. Res. Commun., **1980**, 93, 1238.

[116] Fry, M.; Green, D.E. *J. Biol. Chem.*, **1981**, *256*, 1874.

[117] Hoch, F.L. Biochim. Biophys. Acta, **1992**, 1113, 71.

[118] Lopez-Moratalla, N.; Segovia, J.L.; Santiago, E. *Revista Espanola de Fisiologia*, **1973**, *29*, 329.

[119] Berger, A., Berman, JB, Gershwin, ME. In *Advances in Food and Nutrition Research*; J. Kinsella, Ed.; Academic Press, Inc.: San Diego, CA, **1993**; pp. 260.

[120] Hostetler, K.Y. In *Phospholipids*; J.N.Howthorne and G.B. Ansell, Eds.; Elsevier Biomedical Press: Amsterdam, **1982**; pp. 215.

[121] Wolff, R.L.; Entressangles, B. *Biochim. Biophys. Acta.*, **1991**, *1082*, 136.

[122] Berger, A.; Gershwin, M.E.; German, J.B. *Lipids*, **1992**, *27*, 605.

[123] Paradies, G.; Petrosillo, G.; Pistolese, M.; Di Venosa, N.; Serena, D.; Ruggiero, F.M. *Free Radic. Biol. Med.*, **1999**, *27*, 42.

[124] Ostrander, D.B.; Sparagna, G.C.; Amoscato, A.A.; McMillin, J.B.; Dowhan, W. *J. Biol. Chem.*, **2001**, *276*, 38061.

[125] Petrosillo, G.; Ruggiero, F.M.; Pistolese, M.; Paradies, G. *FEBS Lett.*, **2001**, *509*, 435.

[126] Jemmerson, R.; Liu, J.; Hausauer, D.; Lam, K.P.; Mondino, A.; Nelson, R.D. *Biochemistry*, **1999**, *38*, 3599.

[127] Tsujimoto, Y. *J. Cell. Phys.*, **2003**, *195*, 158.

[128] Tuominen, E.K.; Wallace, C.J.; Kinnunen, P.K. *J. Biol. Chem.*, **2002**, *277*, 8822.

[129] Masden, L., Dyroy, E., Berge, R. In *Current Views of Fatty Acid Oxidation and Ketogenesis: From Organelles to Point Mutations*; Quant, P.; Eaton, S. Ed.; Kluwer Academic / Plenum Publishers: New York, **1999**; pp. 315.

[130] Esposti, M.D.; Erler, J.T.; Hickman, J.A.; Dive, C. *Mol. & Cell. Biol.*, **2001**, *21*, 7268.

[131] Gross, A.; McDonnell, J.M.; Korsmeyer, S.J. *Genes & Development*, **1999**, *13*, 1899.

[132] Van Hoof, C., Goris, J. *Biochim. Biophys. Acta*, **2003**, *1640*, 97.

[133] Stennicke, H.R.; Salvesen, G.S. *Biochim. Biophys. Acta*, **1998**, *1387*, 17.

[134] Bratton, S.B.; MacFarlane, M.; Cain, K.; Cohen, G.M. *Exp. Cell Res.*, **2000**, *256*, 27.

[135] Burgess, J.R.; Stevens, L.; Zhang, W.; Peck, L. *Am. J. Clin. Nutr.*, **2000**, *71*, 327S.

[136] Lim, S.Y.; Suzuki, H. *J. Nutr.*, **2000**, *130*, 1626.

[137] Pawlosky, R.J.; Salem, N. Jr. *Am. J. Clin. Nutr.*, **1995**, *61*, 1284.

[138] Kremer, J.M.; Lawrence, D.A.; Jubiz, W.; DiGiacomo, R.; Rynes, R.; Bartholomew, L.E.; Sherman, M. *Arthritis Rheum.*, **1990**, *33*, 810.

[139] Arm, J.P.; Thien, F.C.; Lee, T.H. *Allergy Proceedings*, **1994**, *15*, 129.

[140] Yokoyama, A.; Hamazki, T.; Ohshita, A.; Kohno, N.; Sakai, K.; Zhao, G.D.; Katayama, H.; Hiwada, K. *Intl. Arch. Allergy Immunol.*, **2000**, *123*, 327.

[141] Rapoport, S.I.; Bosetti, F. *Arch. Gen. Psychiatry*, **2002**, *59*, 592.

[142] Neuringer, N.; Anderson, G.J.; Conner, W.E. *Ann. Rev. Nutr.*, **1998**, *8*, 517.

[143] Saadatian-Elahi, M.; Toniolo, P.; Ferrari, P.; Goudable, J.; Akhmedkhanov, A.; Zeleniuch-Jacquotte, A.; Riboli, E. *IARC Sci. Publ.*, **2002**, *156*, 227.

[144] Wargovich, M.J. *Gastroenterology*, **1992**, *103*, 1096.

[145] Collett, E.D.; Davidson, L.A.; Fan, Y.Y.; Lupton, J.R.; Chapkin, R.S. *Am. J. Physiol - Cell Physiol*, **2001**, *280*, C1066.

[146] de Deckere, E.A. *Eur. J. Cancer Prev.*, **1999**, *8*, 213.

[147] Bagga, D.; Capone, S.; Wang, H.J.; Heber, D.; Lill, M.; Chap, L.; Glaspy, J.A. *J. Nat. Cancer Inst.*, **1997**, *89*, 1123.

[148] Veierod, M.B.; Thelle, D.S.; Laake, P. *Int. J. Cancer*, **1997**, *71*, 600.

[149] Falconer, J.S.; Ross, J.A.; Fearon, K.C.; Hawkins, R.A.; O'Riordain, M.G.; Carter, D.C. *Br. J. Cancer,* **1994**, *69*, 826.
[150] Esteve-Comas, M.; Nunez, M.C.; Fernandez-Banares, F.; Abad-Lacruz, A.; Gil, A.; Cabre, E.; Gonzalez-Huix, F.; Bertran, X.; Gassull, M.A. *Gut,* **1993**, *34*, 1370.
[151] Freedman, S.D.; Katz, M.H.; Parker, E.M.; Laposata, M.; Urman, M.Y.; Alvarez, J.G. *Proc. Natl. Acad. Sci. U. S. A.,* **1999**, *96*, 13995.
[152] Hibbeln, J.R.; Salem, N. Jr. *Am. J. Clin. Nutr.,* **1995**, *62*, 1.
[153] Tomobe, Y.I.; Morizawa, K.; Tsuchida, M.; Hibino, H.; Nakano, Y.; Tanaka, Y. *Lipids,* **2000**, *35*, 61.
[154] Horrobin, D.F.; Glen, A.I.; Hudson, C.J. *Med. Hypotheses,* **1995**, *45*, 605.
[155] McLennan, P.; Howe, P.; Abeywardena, M.; Muggli, R.; Raederstorff, D.; Mano, M.; Rayner, T.; Head, R. *Eur. J. Pharmacol.,* **1996**, *300*, 83.
[156] Dewailly, E.; Blanchet, C.; Lemieux, S.; Sauve, L.; Gingras, S.; Ayotte, P.; Holub, B.J. *Am. J. Clin. Nutr.,* **2001**, *74*, 464.
[157] Kimura, S. J. Pharm. Soc. Jpn., **2000**, 120, 607.
[158] De Caterine, R.; Caprioli, R.; Giannessi, D.; Sicari, R.; Galli, C.; Lazzerini, G.; Bernini, W.; Carr, L.; Rindi, P. *Kidney Int.,* **1993**, *44*, 843.
[159] Leiba, A.; Amital, H.; Gershwin, M.E.; Shoenfeld, Y. *Lupus,* **2001**, *10*, 246.
[160] Fevang, P.; Saav, H.; Hostmark, A.T. *Lipids,* **1995**, *30*, 437.
[161] Wagner, W.; Nootbaar-Wagner, U. *Cephalalgia,* **1997**, *17*, 127.
[162] Bernsohn, J.; Stephanides, L.M. *Nature,* **1967**, *215*, 821.
[163] Crawford, M.A.; Costeloe, K.; Ghebremeskel, K.; Phylactos, A.; Skirvin, L.; Stacey, F. *Am. J. Clin. Nutr.,* **1997**, *66*, 1032S.
[164] Donadio, J.V. Jr. *Am. J. Clin. Nutr.,* **2000**, *71*, 373S.
[165] McGuinness, M.C.; Wei, H.; Smith, K.D. *Exp. Opin. on Invest. Drgs.,* **2000**, *9*, 1982.
[166] Infante, J.P.; Huszagh, V.A. *Mol. Genet. and Metab.,* **2001**, *72*, 185.
[167] Grimminger, F.; Mayser, P.; Papavassilis, C.; Thomas, M.; Schlotzer, E.; Heuer, K.U.; Fuhrer, D.; Hinsch, K.D.; Walmrath, D.; Schill, W.B. *Clin. Invest.,* **1993**, *71*, 634.
[168] Knapp, H.R. World Review of Nutrition & Dietetics, **1994**, 76, 146.
[169] Laugharne, J.D.; Mellor, J.E.; Peet, M. *Lipids,* **1996**, *31 Suppl*, S163.